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~~Reserved for Dr. M. Swaminathan.~~

CONNECTIVE TISSUE SYMPOSIUM

CONNECTIVE TISSUE

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organized by
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FOREWORD

The symposium on 'Connective Tissue' was held in London from July 22nd to 26th, 1956, under the chairmanship of Professor R. E. Tunbridge. The meeting followed the general pattern of previous multi-disciplinary symposia run by the Council. For the first time, however, scientists from Soviet Russia and Hungary accepted our invitation.

It is perhaps appropriate to mention that the C.I.O.M.S. groups some fifty international organizations devoted to the sciences basic to medicine and to the clinical branches. Its aim is to improve communication between disciplines and across national boundaries. Because of its constitution, the Council receives many suggestions for symposia; these come from the international organizations composing its membership, from national research councils or national medical societies and from individuals interested in the Council's work.

Among many topics the various aspects of the biology of connective tissue were suggested for the Council's 1956 symposium. After careful consideration, it was agreed that the meeting would centre on the biochemistry of connective tissue and on the correlations between biochemistry and structure.

The selection of participants was left to the chairman in consultation with the Council's Executive Committee. Every effort was made to make the meeting as representative as possible, both scientifically and geographically. From among the many research workers who, in various countries, are studying connective tissue, a difficult and somewhat arbitrary choice had to be made.

Official languages were English and French, but it soon appeared that English allowed communication between all participants. It is fitting to thank all those who willingly agreed to express themselves in a language which was not their own in order to help the meeting along. In this book, all papers are published in English with the exception of the one by Dr. Delaunay and Mlle Bazin but a summary in English follows the paper.

All discussions were immediately transcribed from magnetic recordings by Dr. Geoffrey Wood and Dr. Madeline Keech to whom thanks are due. The discussions are printed in précis form

at the end of each paper. Papers and discussions are to be taken together just as they were delivered and discussed. Much of the cut and thrust of debate has necessarily been lost for which we are sorry – but the irrelevant and the repetitious has been eliminated in order to present the reader with the substance of the discussion.

We hope that this monograph will be of use to all who study the biology of connective tissue.

INTRODUCTION

W. T. Astbury

In nature's infinite book of secrecy
A little I can read.
Antony and Cleopatra, Act I, Scene 2.

When our Chairman kindly invited me to contribute a general introduction to this symposium and presented me at short notice with a formidable collection of summaries of papers to be contributed by the other participants, my first reaction, I confess, was to recall Sydney Smith's famous remark: 'I never read a book before reviewing it; I find it prejudices the mind.' Here now, I thought, was a beautifully analogous situation where to follow Smith's example was most strongly indicated. I have compromised though – and in the way in which I suppose I was expected to; that is to say, I have of course read the summaries, but this brief assessment of how we stand at the moment with regard to the connective tissue problem is for the most part only as I see it personally. Which means also that the bad parts are mostly mine too.

We would all agree, I believe, on the convenience of subdividing our subject, as has been done for the purposes of this symposium, under the headings: Cells, Ground Substance and Fibres; yet at the same time we all probably have a deeper tendency to think of the connective tissue system as a kind of unit – a combination that is to a first approximation self-contained and indivisible. We imagine the cells manufacturing a fundamental matrix, which then acts as site and precursor for the fibre complex that is the principal, and relatively stable, end-product of the system. And always, *ex hypothesi*, we search for common factors, common manifestations. The chief motive in fact of a meeting such as this must be, directly or indirectly, consciously or unconsciously, to inquire again what are these common features and to examine once more how far we have gone in their characterization.

Since it is still very much biogenesis and the primary synthetic steps which are the most difficult and mysterious, the procedure of least resistance in molecular biology generally, and in the connective tissue problem in particular, is to work mostly backwards from the

more permanent end-products – in this case the fibre complex of collagen, elastin, reticulin and anything else there may be, on which indeed the idea of connective tissue unity is just now most clearly focused. This feeling for a common plan underlying the fibrous end-products of the connective tissue system derives from the molecular level of X-ray diffraction analysis, which first brought about in pre-war days an astonishing simplification among the apparently enormous diversity of fibrous proteins. It was found that, in terms of polypeptide-chain types, there are in the main only two configurational schemes, one comprising the long-range elastic fibres of the keratin-myosin-epidermin-fibrinogen group (k-m-e-f group), and the other the inelastic fibres of the collagen group. The collagen group was named after its most familiar member, orthodox collagen of classical histology, but perhaps a little deceptively, because it transpired that one of the most impressive things about the group was its very wide range, including as it does numerous structures previously considered to have little or nothing to do with one another – for example, white connective tissue fibres, tendon and cartilage, the scales and fins (elastoidin) of fishes, the ichthyocol of swim-bladders, the cuticles of Annelid worms, the filaments ejected by the sea-cucumber, jelly-fish, to quote only a selection. They all (and gelatin too) give the same distinctive kind of large-angle X-ray fibre diagram, quite different from anything found with the k-m-e-f group, and this, the diffraction expression of the peculiar polypeptide-chain configuration they all have in common, still remains the only sure criterion yet discovered of their fundamental family relationship. Many of them give also the collagen small-angle X-ray diagram, corresponding in period (about 640 Å) to the bands seen in the electron microscope, but this is not an essential but is associated with the next stage of organization: the small-angle X-ray diagram is easily destroyed, and the bands seen in the electron microscope can also be altered or destroyed (there are none in any case in the fibrils of the earthworm's cuticle), but the large-angle X-ray diagram persists.

Here, beyond doubt, is one of the major designs of molecular phylogeny, comprehensive enough to take in the connective tissue fibres in its stride, so to speak – that is, potentially; for whether it does so actually, and completely, is not yet demonstrated. I myself feel that it is an eminently plausible view that the connective tissue combination should represent a sort of pocket of unity within the

greater unity; that, in other words, it should constitute a specialized subgroup of the collagen group something like the feather-keratin subgroup of the k-m-e-f group. After all, now that the reticulin fibres have been found to satisfy basic collagen-group requirements, only elastin remains to be accounted for, and even that too, these days, is at last beginning to show signs of conforming.

I think, therefore, (the odds in support of the suggestion being at least two to one), that I might very usefully devote much of the rest of this introduction to summarizing some of the findings and conclusions lately arrived at regarding that innermost question of the collagen group from which all else probably spreads out automatically. I refer to the constitution and curious polypeptide-chain configuration which gives rise to the characteristic X-ray diagram and which has offered such long resistance to attempts at elucidating it. This really is the central problem; for let me emphasize that the master plans of the fibrous proteins revealed by X-ray analysis are type-specifications, or themes, susceptible of many variations; and once we have grasped what the minimum demands of the collagen group are, we ought then to be able to proceed to correlate the various stages or ramifications, complete or incomplete, of subsequent building-up processes – 'procollagens' and 'tropocollagens' and concepts of that kind – with comparative ease, or at any rate much more confidently.

The new outlook in recent years on possible polypeptide-chain configurations is inspired, as everyone knows, by what may be called the helical interpretation of Pauling and Corey, according to which the unstretched α-form of the k-m-e-f group (and of various synthetic polypeptides), for example, is based on a helix comprising approximately 3.6 amino-acid residues per turn, each residue occupying a length in the direction of the fibre axis of about $1\frac{1}{2}$ Å. (This is only the bare bones of the k-m-e-f story; the complete explanation is an elaboration that has not yet been fully worked out.) In the β-form of the k-m-e-f group the polypeptide chains are pulled out almost straight, so that the average length of an amino-acid residue in the direction of the fibre axis is now approximately 3.33 Å, and the structure built up is very probably a certain one of the combinations of extended chains that Pauling and Corey have called 'pleated sheets'. We need not, here, consider the k-m-e-f group in any further detail, but I want to make the point that, of the two principal configurations I have just mentioned, the 3.6 α-helix (it is one of a

series of analogous helices) is held in that shape by hydrogen bonds between CO and NH groups all belonging to the same helix – they are all *intra-chain* bonds; while the β-pleated sheet is held together by hydrogen bonds linking CO and NH groups in adjacent chains – they are now all *inter-chain* bonds. What other possibilities are there that might be identified with the collagen configuration, which, as I have said, is so curiously distinct from either of these two? After the triumph of the α- and β-configurations it would seem not too difficult to advance thence on collagen and, with the aid of the elegant diffraction treatment given by Cochran, Crick and Vand (1952), to clear it up similarly, but as a matter of fact it has proved surprisingly obstinate. Ever since the early days when the collagen group was first recognized, we at Leeds have argued that the collagen chains are practically inextensible for special stereochemical reasons connected with the preponderance of proline and hydroxyproline residues; that actually they are *shorter* than in the β-configuration to the extent that the length per residue in the direction of the fibre axis has now fallen to as low as 2.86 Å; but what exactly was the nature of this constriction? That was always the question; and even with the advent of the new helical ideas, as applied by Pauling and Corey themselves, by Bear, and by Randall and coworkers, for instance, it still turned out far from easy to decide what particular form of helix or combination of helices best agreed with all the X-ray data (recently made more precise by improved diagrams at the hands of the Randall school) and supporting observations.

The break came eventually through papers by Ramachandran and Kartha (1954, 1955, 1956), Cowan and McGavin (1955), Cowan, McGavin and North (1955), Crick and Rich (1955), and Rich and Crick (1955). What it amounts to is roughly this, that Ramachandran and Kartha first proposed a structure along the desired lines, and it has seemingly been improved and rationalized, so to say, by the other authors in the light of X-ray analyses of poly-L-proline (Cowan and McGavin) and of the hitherto obscure crystallographic modification of polyglycine (Crick and Rich) that has been named polyglycine II (in polyglycine I the polypeptide chains are in an extended β-configuration). It was found that in poly-L-proline and polyglycine II the backbone configuration is the same – a helix with three residues per turn, each residue occupying a length of about 3.1 Å (though the six-fold system of

inter-chain CO. . .NH hydrogen bonds in polyglycine II is perforce absent from the polyproline chains of imino residues); and this striking result, on top of the famous meridional reflection at 2.86 Å and the well-known piece of chemical analytical information that one-third of collagen consists of glycine residues and at least another quarter of proline and hydroxyproline residues, then led irresistibly to the conclusion that the collagen structure is simply the polyglycine-polyproline scheme adapted to a constitution that is neither one thing nor the other yet includes quite a lot of both. It is based not on the straight trigonal helix but on a slight helical distortion thereof, a so-called 'coiled coil'.

One takes a triad of helices from the polyglycine-polyproline structure (there are two ways of doing this) and twists the three chains slowly round one another so that the number of residues per turn, with respect to the common axis, becomes $3\frac{1}{3}$. The period is 2·86 Å and corresponds to three turns. So at last our original supposition that 2.86 Å, the spacing of the meridional reflection so characteristic of the collagen diagram, measures the length per residue in the direction of the fibre axis, is justified.

The answer to the question I put a moment ago as to what other formal description, in contrast to the two appropriated to the α-helix and the β-pleated sheet of the k-m-e-f group, is available for the collagen group is that the collagen configuration is at heart (that is, before adaptation) a helix held in that shape by inter-chain bonds; which means that, formally again, it also falls after all into the same class as the β-configuration, which is geometrically a helix with two residues per turn. From an outside viewpoint, though, the eventual structure, that is after adaptation of the polyglycine-polyproline scheme to the requirements of the betwixt-and-between chemical constitution, could be described as belonging in a sense to the class of helices, e.g. the α-helix, held in shape by intra-chain bonds; for the inter-chain bonds linking the three initially trigonal helices are the intra-chain bonds of the compound major helix into which they are finally twisted.

The kinds of inter-chain bonds that hold the primary helical unit of the collagen structure in that shape, and indeed go further and result in the end in a triad of such helices becoming also twisted round one another to form a coiled coil, are still under debate, though obviously some must be CO. . .NH hydrogen bonds and others may well be hydrogen bonds of the type postulated by

Gustavson (our much-respected doyen of the Collagen Group!) as linking the hydroxyproline OH group with a CO group on an adjacent chain. Incidentally, we must beware of perhaps misapplying Gustavson's idea, which is a deduction from the properties of *collagens* – collagens of different hydroxyproline contents. It is concluded that the bond confers resistance to thermal shrinkage and one might expect, therefore, to solution; but gelatin is soluble in warm water yet has the same hydroxyproline content and gives the same kind of large-angle X-ray diagram as collagen – the diagram, that is, from which the coiled-coil structure has been derived; and the earthworm's cuticle gives the same kind of diagram too, though, as first shown by Singleton (1955) in our laboratory at Leeds, it has the highest known hydroxyproline content (equivalent to 14.6 per cent of the total nitrogen) combined with the very low thermal-shrinkage temperature of 32-33° C and begins to dissolve in water at about 40° C. Thus, of the two current crystallographic interpretations of Gustavson's bond: (*a*) that it links together the components of the triads, and (*b*) that it links the triads to one another, it would seem that the latter is the more plausible – without necessarily excluding the former entirely, nevertheless, because another, and contrasting, consideration not to forget is the fact that the normal large-angle diagram of collagen disappears during thermal contraction and is replaced by an 'amorphous' diagram.

Progress in sorting out the details and permissible variations within the collagen framework depends at the moment more on chemistry than on physics – this constant swinging of the research pendulum is a notable and characteristic feature of modern biomolecular studies – and keen investigations to that end are being carried out these days in more than one laboratory, investigations to determine not merely the proportions of the amino-acid residues but also their order. Already such findings as, for example, the common occurrence of the sequence -prolyl-hydroxyprolyl-glycyl- make it seem highly unlikely, because of insufficient amino residues, that the construction is uniform throughout, and it could be, therefore, that either the helices of a triad are not alike, or there are different kinds of triads or of still larger (filamentous) units, or all these things together – not to mention something else that is often a skeleton in the macromolecular X-ray cupboard: I mean the possibility that what is giving rise to the regular diffraction pattern represents only part of the structure under examination; for instance, more uniform and

crystallographically better-fitting sections of long chains that for the rest are not so adaptable, or limited groups of whole chains that are better crystallizable. Anyhow, it will be appreciated that there is plenty of scope (perhaps a little too much for present comfort) in what is now known of the molecular framework of the collagen group, including most if not all of the connective tissue fibres, to satisfy gradation and variability requirements. A great deal of difficult, painstaking work remains, to be sure, but I have the feeling that it really is all becoming more and more a question of detail than of principle. The outlines of the plan are now very nearly in focus, and I find that most inspiriting.

It follows that the main burden and responsibility of connective tissue research devolves more than ever on the procedures and results of *extraction*. The business of extraction and separation dominates biological chemistry and physics – it 'delivers the goods' – and here, in the needs of disentangling the connective tissue complex, we see a particularly fine illustration, in an exciting configurational setting too. Components are made available conforming in the first place to certain minimum structural specifications now partly recognizable, and these are then made use of, with such modifications as may be required and are permissible, in successive stages and paths of increasing organization, diversified maybe from time to time with degradational episodes also. The task is to complete the recognition of these components and their modifications and combinations by catching them on their way up or down and looking at them separately, and it is one of the most worthwhile in medical science.

And to put the aim again at its least complicated, most direct – never mind at this juncture whether too simple, too direct – it is to identify an ideal or limiting building-stone, say the tropocollagen of the F. O. Schmitt school or Orekhovitch's procollagen, or, not improbably, a precursor of both, incorporated in an ideal or limiting edifice, such as a regular aggregation of the X-ray analyst's triple coiled coils. The present incomplete and imperfect correlation between molecular form and chemical constitution is, however, symbolic and perhaps more representative of the actual state of affairs, in which the ideal plan, though governing in principle all the fibrous products, yet impresses itself to widely varying extents and, indeed, succeeds sometimes only so very indifferently as effectively to lose its identity.

Which brings me now, *via* this last remark, to a reconsideration of the apparently outstanding case of elastin – 'reconsideration', because I proposed as long ago as before the war that elastin might very well be a member of the collagen group but with a thermal contraction temperature below ordinary temperatures, and in the light of the chemical and structural attainments of the time we studied the matter by X-rays and obtained the first diffraction diagrams of genuine collagen-free elastin, unstretched and stretched (Astbury, 1938, 1940). More complete chemical evidence (see, for example, Tristram, 1953) has since made it clear that, though the glycine and proline contents of elastin are much the same as, if not even a little higher than, in orthodox collagen, there is a remarkable decrease in the proportions of the polar residues, not only basic and acidic but most noteworthy of all, hydroxyproline. We are therefore at once put in mind again of Gustavson's suggested inter-chain linkage between a peptide CO group and the hydroxyproline OH group, which reveals itself specially in its tendency to inhibit thermal contraction, and in this context elastin certainly looks like a direct extrapolation from collagen through lower and lower hydroxyproline contents. The effect of the loss of the stabilizing influence of the hydroxyproline linkages will be enhanced by the substitution of non-polar for most of the other polar side-chains besides, which will both eliminate more of the stronger cross-linkages and further lower the thermal contraction temperature by a process of 'internal plasticization', in the manner well known to high-polymer physical chemistry, where, in addition to the method of increasing plasticity (molecular mobility) by incorporating small *extraneous* molecules between the long chain-molecules, an impressive 'opening-up' can often be brought about by the artificial introduction of irregular side-chains in the main-chains themselves.

In the same connection one cannot help wondering about the relation of the configuration of polyglycine II to temperature. According to Meggy and Sikorski (1956), polyglycine has a transition temperature round about 60° C: when polyglycine I is dissolved in saturated calcium chloride solution then re-precipitated by addition of water, the precipitate at ordinary temperatures is polyglycine II, but between about 60° and 100° C it is a mixture of polyglycine I and II. The configuration is thus not directly dependent on the mode of chemical synthesis, but the fact that the helical form which is more stable at ordinary temperatures is the one simulated

and adapted in the construction of the collagen complex – that, and the occurrence of polyproline also in the same form, and that form only, presumably – may conceivably be found to have significance later on when the deeper biogenetic details begin to come in sight. For the present it is worth a mental note that in this context too, elastin is a sort of extrapolation from an intermediate state of folding.

The X-ray diagram of elastin is again a kind of extrapolation – a not unreasonable extrapolation from the diagram given by thermally-contracted collagen; for the main halo is in about the same position (4.4-4.5 Å) in both, but elastin has also a faint halo at something over 9 Å, i.e. in the neighbourhood of the 'side-chain reflection' of the k-m-e-f group, whereas there is a striking emptiness inside the main halo of thermally contracted collagen. May it not be that the coiled-up state and rubber-like elasticity normal to elastin betrays in this way its affinity with at least the manifestation of collagen which, though abnormal, is also coiled-up and rubber-like, while at the same time throwing out a hint that the new (mostly non-polar) side-chains encourage a certain amount of folding held in shape by *intra-chain* hydrogen bonds?

If only for these reasons, 'I think', to quote from my Procter Memorial Lecture (1949), 'that we still ought to work on the hypothesis that elastin is at least closely related to the collagen group'. But they are not the sole considerations: for instance, *à propos* of what I have just said about the X-ray evidence, was my phrase 'though abnormal' a justifiable description of the coiled-up, long-range elastic manifestation of collagen? It is natural to look first and foremost for the link, if such there be, between elastin and the most finished, most stable, most 'normal', and best documented manifestation of collagen, but it is important not to be obsessed in this regard but to remember too that the coiled-up state of collagen can be brought about not exclusively by raising the temperature in the presence of water but also by the action of certain swelling solutions even at ordinary temperatures. In developing tissues, which are so much more heavily swollen than the finished products, the abnormal may therefore be the normal, as it were, and immature collagen and elastin may be nothing like so different as they come to be in subsequent phases of growth.

And then, as intriguing a set of observations on possible collagen-elastin interrelationships as have been reported for a long time, there are the recent joint studies (Burton *et al.*, 1955) undertaken in the

University of Leeds by the Nuffield Gerontological Unit and the Departments of Medicine and Leather Industries. It is found that the action on collagen fibrils of various chemical reagents (including enzymes) at 37° C results in a wholesale conversion into elastin-like structures as judged by appearances in the electron microscope and by histological staining tests; and what is more, when alkaline buffers are forced through pads of collagen, the percolates are found to be very rich in hydroxyproline and arginine, the two amino acids conspicuously deficient in elastin as compared with collagen. Were it not for this last finding one might be inclined to look askance at what seems to be shown in the electron microscope and by classical staining techniques, but all three kinds of results together make one think twice. Strictly speaking, all that the experiments demonstrate unambiguously as yet, is that the collagen complex can be broken down in such a way as to lead to preferential extraction of hydroxyproline and arginine – and this in itself is not unexpected, either, in view of the combined X-ray and chemical analytical indications discussed above – but let us hope they mean something far more gratifying. They must be followed up unremittingly, it goes without saying, and every effort made to prove, by X-ray and elasticity tests for example, that the man-made 'elastin', besides looking and staining like natural elastin, *is* the same sort of thing as natural elastin, as near as makes no matter.

All in all, it is not too bad a case for tentatively accepting elastin as a bona fide if somewhat surreptitious member of the collagen group, and I repeat that I personally like the idea.

The final answer goes back, of course, to the biosynthesis of the polypeptide chains and reactions about which we are abysmally ignorant. After these unknown initiating steps the evidence then points mainly to extracellular mechanisms, in the so-called ground substance; though indications are not lacking that the cell surface, if not still also the cell interior, plays its part too. The overall impression is of precipitation processes under more or less remote control – less say in the case of the crossed fibrils of the earthworm's cuticle fabricated on a layer of epithelial cells, and more say in the case of the threads ejected by the sea cucumber. The collagen group is truly 'of most excellent fancy'!

It would hardly be correct, I suppose, though stylistically tempting, for me to leave it at that and now without further ado to bring this Introduction to a close. I appear to have said nothing yet about

polysaccharides, and no talk on the collagen group and connective tissue may possibly be considered adequate without at least reciting once more our article of faith that their synthesis and general activities are inseparably bound up with the participation of certain special polysaccharides. I am afraid though, that having said that, it is still baffling to give any real body and precision to the concept; for these polysaccharides are among the most self-effacing molecular collaborators imaginable: the nearer their job is to completion, the fewer the stoichiometric signs that they ever had anything to do with it. In the end they are rather like the Cheshire Cat – only their grin remains.

ON THE STRUCTURE AND FUNCTIONS OF THE MAST CELL

G. Asboe-Hansen

Mast cells are large connective tissue cells with an approximately central nucleus and ample cytoplasm with more or less densely arranged granules. Their shape and size show considerable variation. The granules are evenly distributed over the cytoplasm and stain metachromatically with certain basic dyes.

Histochemical, chemical and histophysiological studies have led to various theories regarding the function of the mast cells:

(1) In 1937 Holmgren and Wilander advanced the theory that the mast cells contain and form heparin. Jorpes, Werner and Åberg (1948), and Friberg, Graf and Åberg (1951) have demonstrated that the granular substance of the mast cells is not di- or tri-sulphated, but that it may be monosulphated, and may be the precursor of heparin.

(2) In 1950 I performed a series of experiments which led to the theory that mast cells may produce hyaluronic acid, perhaps by way of a heparin-like precursor and under some hormonal influence.

It seemed obvious that heparin production could not be the only task of the enormous mass of mast cells in the body. Searching for the origin of the mucopolysaccharides of the connective tissue ground substance, I realized that the mast cell is the only connective tissue cell which has been shown to contain mucopolysaccharide material. Morphological and histophysiological studies compared with chemical data indicated that the material released contains hyaluronic acid.

(3) In 1953 Riley and West advanced the theory that the mast cells contain histamine which can be released to the tissues. Since then several authors have stressed the parallelism between the mast cell content of a tissue and its histamine value (Riley and West, 1953, 1955; Cass *et al.*, 1954; Benditt *et al.*, unpublished). In 1955 Riley and his co-workers could not find conformity between the liberation of histamine and heparin in rats, though disruption and degranulation of the mast cells occurred in response to injection of the chemical histamine-liberator substance 48/80.

Hormones have been shown to influence the mast cells and the mucopolysaccharides of the connective tissue ground substance.

It should be emphasized, however, that the experiments and findings that have led to the theories regarding the functions of the mast cells, the formation of mucopolysaccharide, the formation of histamine and the hormonal influence on the cells relate to *fixed* tissues, that is, dead cells. Since fixation and preparation inevitably involve certain sources of error, and incidentally the same applies to freeze-drying, studies of living connective tissue with intact circulation might be expected to afford information of value in this discussion.

I. STUDIES ON MAST CELL REACTIONS IN LIVING CONNECTIVE TISSUE OF THE HAMSTER CHEEK POUCH

These investigations were performed in collaboration with a Finnish colleague, Dr. Otto Wegelius, working in my laboratory. These experiments have led to a view which unites the different theories and assigns to the mast cells an acceptable position in relation to mucopolysaccharide as well as histamine. In addition they have afforded some information regarding the influence of hormones upon the cells.

The method has previously been described in detail by Wegelius and Hjelmman, 1955. The cheek pouch of the hamster allows *in vivo* studies of hormonal effects on the mast cells.

The cheek pouch is drawn out, the upper layer carefully incised and the membrane stretched out by means of needles over a hole in a disc of cork. If desired this may be fitted into a moist chamber of plexiglass containing circulating physiological saline at body temperature. The mast cells are studied by transmitted light by means of water immersion lenses as well as by incident illumination. The thin membrane getting intact circulation was studied unstained by the phase contrast microscope, and, after staining, by bright field microscopy. Toluidine blue in physiological saline, 1:100 to 1:100,000, was dropped on the exposed connective tissue surface of the membrane. The weak dilutions do not appear to be toxic to the mast cells, as degranulation and intracellular rearrangements of the granules may be observed after staining. Strong solutions, on the other hand, apparently stop the function of the cell immediately. This confirms the findings of Grossfeld in tissue cultures (Grossfeld, 1954).

The animals are anaesthetized by intraperitoneal injection of 0.08–0.15 ml. of a 6 per cent nembutal solution.

Hormones

The systemic treatment with various hormones was given in the form of injections into the femoral muscles, and the topical treatment as injections into the connective tissue between the two epithelial layers of the cheek pouch.

TABLE I

SYSTEMIC TREATMENT
(intramuscular injections)

Hormone	*Dose*	*Time of action*	*Results*
Corticotrophin	5+5 mg./day	7 days	Degranulation, clumping, vacuolation
Cortisone acetate	10 mg./day	5 days	Degranulation, clumping, vacuolation
Thyrotrophin	1 USP. unit/day	5 days	Numerous well-granulated cells
Thyroxin	0.5 mg./day	5 days	Degranulation, small perivascular cells, intact
Somatotrophin	0.5 mg./day	20 days	Numerous well-granulated cells
Controls	Daily injection of physiological saline solution		

TOPICAL TREATMENT
(injections into the connective tissue of the extended cheek pouch)

Cortisone acetate	0.25 ml. = 6.25 mg.	38 hours	Degranulation, clumping, vacuolation
Hydrocortisone acetate	0.25 ml. = 2.5 mg. and 0.5 ml. = 5 mg.	5 hours and 38 hours	Degranulation, clumping, vacuolation
Hydrocortisone tertiary-butyl-acetate	0.5 ml. = 12.5 mg.	38 hours	Degranulation, clumping, vacuolation
Controls	0.25–0.5 ml. physiological saline sol. or Tyrode sol.		

The results were as follows:

Corticotrophin and *cortisone acetate* have been found to exert an action on the mast cells in the cheek pouch after systemic treatment whilst cortisone acetate, *hydrocortisone acetate* and *hydrocortisone-tertiary-butyl-acetate* also act on topical injection. The cells become degranulated, the granules gathering into clumps, and vacuolation is not uncommon. After topical injection of cortisone acetate, hydrocortisone acetate, and hydrocortisone-TBA into the cheek pouch, the mast cells can no longer be identified in the immediate vicinity of the depot. Farther away mast cells are visible, but greatly changed showing large vacuoles and clumping of the granules (Fig. 4). A special phenomenon, viz. mast cells with large, round, ortho- or slightly metachromatic granules or drops, abound in the specimen.

Inspection of connective tissue after intramuscular *thyrotrophin* injections reveals well-granulated mast cells in large numbers.

Cheek pouches of animals treated with *thyroxin* showed a remarkable appearance. A considerable number of *small* mast cells were observed, primarily situated perivascularly. In the connective tissue, farther away from the vessels, mast cells were few and in various stages of degranulation. Clumping of the granules was quite a common phenomenon.

Following intramuscular injection of *somatotrophin*[1] the connective tissue exhibits large, well-granulated mast cells in large numbers, like the finding in animals treated with thyrotrophin.

Control experiments were performed to ascertain the possible effect of the dye and manipulations.

We extended these investigations to include studies on mast cell responses to other agents in order to elucidate the functions of the cells.

Histamine

There exists much evidence that at least some mast cells contain considerable amounts of histamine; but the problem whether the mast cell changes observed after injection of histamine-liberator

[1] *Preparations:*

Adrenocorticotrophic hormone = Acton, Frederiksberg Chemical Factories Ltd., Copenhagen.

Cortisone acetate = Cortone acetate, Merck & Co., Inc., New York, U.S.A.

Thyrotrophic hormone, the Armour Laboratories, Chicago, Ill., U.S.A.

Thyroxin, Hoffmann-La Roche & Co., Basel, Switzerland.

Hydrocortisone acetate = Hydrocortisat, Leo Pharmaceutical Products, Copenhagen.

Hydrocortisone-tertiary-butyl-acetate = Hydrocortone, TBA, Merck & Co., Inc., New York, U.S.A.

Somatotrophic hormone, the Armour Laboratories, Chicago, Ill., U.S.A.

substances are a specific response in some way bound up with the liberation of histamine, or a response to the tissue effects of the histamine liberated from other sources, is still unsolved.

The aim of the following experiments was, therefore, to study – under conditions as physiological as possible – the response of *living* mast cells to histamine as well as to histamine-liberators in connective tissue receiving unhindered blood supply and on the whole left largely intact.

The effects of distant injections of a neutral and isotonic solution of histamine hydrochloride, of compound 48/80 in physiological saline solution, of stilbamidine isethionate (M. & B.) in sterile water, of peptone (Witte) in physiological saline, and of sterile water were tested. Stilbamidine is highly toxic; intracardiac injections were given very slowly in order to avoid immediate death. Compound 48/80[1] is a more specific histamine-liberator of lower toxicity. Water induces an osmotic imbalance and is found to liberate histamine locally (Fawcett, 1954).

Both cheek pouches of a hamster were examined, extended over two holes in a disc. As a control the left cheek pouch was examined before the distant injections of the mentioned agents. The connective tissue of the divided control pouch was stained for 5 minutes with 1 per thousand toluidine blue in physiological saline. Thereafter, as indicated by Riley, shock doses of the agents were injected intramuscularly, intraperitoneally, or intracardially. The concentrations of the solutions are listed in Table II.

TABLE II

INFLUENCE OF HISTAMINE AND HISTAMINE-LIBERATOR SUBSTANCES ON LIVING TISSUE MAST CELLS

Agent	*Conc.*	*Dose*	*Mast cell response*
Histamine HCl.	1 mg./1 ml.	0.5-2 mg.	Degranulation, clumping
Cp. 48/80	1 mg./1 ml.	0.4 mg.	Degranulation, clumping
Stilbamidine isethionate	10 mg./1 ml.	7.5 mg.	Degranulation, clumping
Peptone	100 mg./1 ml.	50-100 mg.	Degranulation, clumping
Sterile water		2 ml.	Degranulation, clumping, disruption

Agents administered by intracardiac, intramuscular and intraperitoneal injections. The observation periods were 3, 15, 30, 40 and 60 min.

[1] A polymeric condensation product of p-methoxyphenethyl-methylamine and formaldehyde, kindly supplied by Burroughs Wellcome & Co., England.

The effects were studied in the right cheek pouch, handled and stained in exactly the same way as the control pouch.

A few minutes after the injections of histamine and histamine-liberators the connective tissue of the cheek pouches looked more or less oedematous.

Distinct, and often extremely marked degranulation of the mast cells was observed following systemic administration of shock doses of histamine (Fig. 2) as well as of the histamine-liberators 48/80 and stilbamidine. The effect on the cells of compound 48/80 was observed to set in within 5 minutes after the injection, and to reach a maximum within 30 minutes. Peptone induces slight degranulation, but never as marked as the above-mentioned agents. Following systemic administration of sterile water some cells were irreversibly disrupted, but most cells remained intact, though to some extent degranulated. Clumping of the granules was noticed in some of those cells which still retained their granules. There were occasional cells with apparently normal granulation (Fig. 1), or even groups of such cells, although almost exclusively around the larger blood vessels. By contrast, pronounced degranulation was always observed around the capillaries, small arterioles and venules. Velocity of the changes appeared to depend on dosage.

In the control experiments involving systemic administration of physiological saline and Tyrode solution similarly applied, and in the same amounts, degranulation was not more marked than in the directly stained specimens.

When studying mast cells visualized in the phase contrast microscope without staining, we were able to observe consistent changes in the mast cells after injection of histamine and histamine liberators. The cell contours became indistinct, and the granular structure was altered; rearrangement of the granules and vacuolation were observed; sometimes the pattern was entirely blurred. Apparently unaffected mast cells were also nearly always demonstrable by this method.

Serotonin (= 5-hydroxytryptamine)

Serotonin creatinine sulphate (National Biochemical Corp.) injected intraperitoneally into hamsters in doses of 10 to 20 μg. per gramme body weight induced pronounced oedema of the connective tissue. A considerable degranulation of the mast cells in the

cheek pouches was observed within 10–30 minutes. Irreparable breakdown of the cells, was observed only in exceptional cases.

Mast Cells and Tissue Water

The following series of experiments is concerned with the *local* effect of various fluids upon connective tissue mast cells. One of the two cheek pouches of the hamster was invariably used for control. Sterile water, physiological saline solution, Tyrode solution, 2 per cent saline solution, solutions of hyaluronidase and of hyaluronic acid were injected between the two layers of the cheek pouch or dropped on to the lower membrane of the outspread split pouch. In order to approach physiological conditions as far as possible, the hamsters were placed on a warming stage mounted on the microscope. One of the hyaluronidase solutions used contained 100 viscosity reducing units per ml. and another, highly concentrated

TABLE III

INFLUENCE OF VARIOUS FLUIDS ON LIVING TISSUE MAST CELLS

Fluid	*Changes in mast cells within 30 min.*
Sterile water	Degranulation, disruption, granule swelling
Physiological saline	Degranulation
Tyrode sol.	Degranulation
2 per cent saline sol.	Shrinkage
Hyaluronidase in physiological saline (100 and 1000 VRU/ml.)*	Rapid degranulation
Hyaluronidase in Tyrode sol. (100 and 1000 VRU/ml.)	Rapid degranulation
Hyaluronic acid 1 and 2 per cent in physiological saline	Insignificant changes
Traumatization (tissue edema)	Degranulation, disruption, granule clumping

* Viscosity reducing units.

one 1000 V.R.U./ml., the last mentioned preparation contained 3000 V.R.U./mg., or about 27,000 V.R.U./mg.N.[1]

Hyaluronic acid, produced from bovine vitreous humour and from human umbilical cord[2] was applied – in 1 per cent and 2 per cent solutions.

In order to obtain a local tissue oedema by mechanical trauma the cheek pouch was rubbed a few times between the thumb and the index finger.

Injection of the fluids into the connective tissue of the cheek pouches invariably caused disruption, i.e. irreversible damage, of some mast cells. Perceptibly fewer disintegrated cells were seen after the dropping procedure.

After application of water a considerably larger number of cells in various stages of degranulation were observed than in the directly stained control pouches. In some of the cells the granules appeared swollen. Many granules were found lying free in the connective tissue ground substance after disruption of the cells, still showing intense metachromasia; they retained their colour unchanged for hours. Varying numbers of macrophages containing in their cytoplasm metachromatic granules were observed, a phenomenon which was interpreted as evidence of phagocytosis of emitted mast cell granules. These granules retained their metachromasia for some time within the bodies of the phagocytizing cells (Fig. 3).

After treatment with physiological saline and Tyrode solution a considerable increase in the number of degranulated cells was observed as early as 20-30 minutes after application. These cells were fairly often surrounded by a zone of intercellular substance showing more intense metachromasia than more distant areas. Entirely degranulated cells showed a basic pattern like a honeycomb or sponge (cf. Fig. 2).

Thirty minutes after application of a 2 per cent saline solution the mast cells revealed less degranulation than in the control pouches treated with physiological saline. The cells were smaller and their granular structure seemed to be denser.

Hyaluronidase caused marked degranulation occurring even within 10 minutes, at which time the control specimens still had well-granulated cells. Even in these specimens a few cells were well stained and metachromatic after a fairly long time of treatment.

[1] Kindly supplied by A/B Leon, Hälsingborg, Sweden.
[2] Kindly supplied by Wyeth International Ltd., Philadelphia, Pa., U.S.A.

These cells may have been protected by adipose tissue, thicker fibrous layers, etc. The granules remaining in partially degranulated cells showed normal metachromasia. The size of the cells was not definitely altered. No extracellular metachromasia was noticed, nor any signs of phagocytosis.

Hyaluronic acid, 1 per cent and 2 per cent in Tyrode solution, did not induce any significant changes of the mast cells within 40 minutes. The cells stood out intensely metachromatic and densely granulated (cf. Fig. 1). Later on a slow degranulation took place.

After mechanical traumatization of a cheek pouch mast cells with conglomerated granules and more or less degranulated were observed in considerably larger numbers than in the control pouch; in addition, a relatively large number of disintegrated cells, as well as macrophages with phagocytized granules.

DISCUSSION

It is obvious that the granule substance may come into the ground substance, either by a *disruption* caused by some violent – physical or chemical – action during which the cell perishes, or by a physiological *degranulation*, probably involving some alteration of the granule substance, e.g. with regard to water solubility, before or during the passage from within to outside the cell.

Studies of living connective tissue of the hamster under the influence of hormones revealed morphological changes of the mast cells, largely identical with those seen in dead, fixed tissues of other animals and humans similarly treated. It is evident that the extremely marked and consistent changes observed must reflect an interference with the functions of the mast cells.

The degranulation of the mast cells invariably seen after administration of histamine and histamine-liberators shows that the mast cells of the hamster are sensitive to the influence of both. The observation that histamine exerts an action as powerful as that of the histamine-liberators, indicates that the mast cell changes are an unspecific response to the *effect of* histamine on tissues rather than a specific response to histamine-liberators.

When disintegration of the cell occurs the contents are released; consequently, if the mast cells do contain histamine this substance must also be released. Whether degranulation, which is a function of the living cell, entails a release of histamine together with the metachromatic substance, still remains an open question.

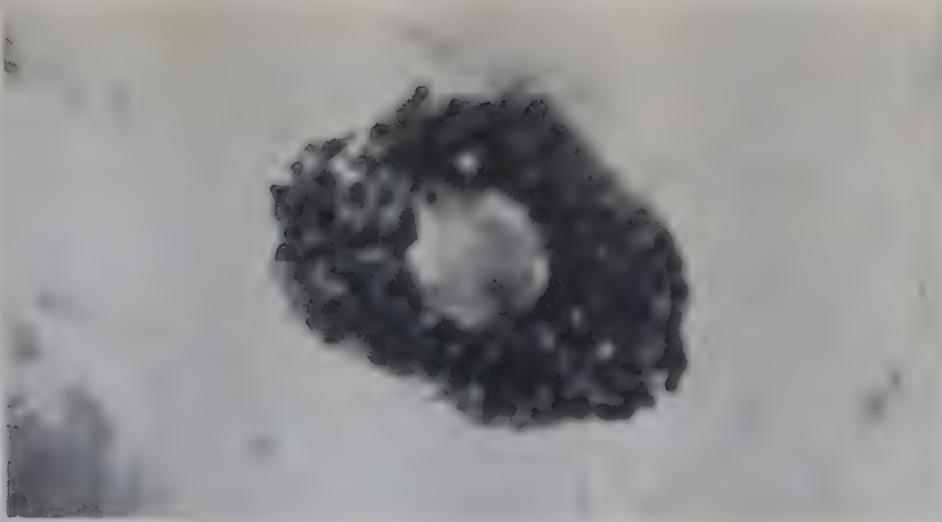

Fig. 1
Normal mast cell loaded with metachromatic granules. Staining: toluidine blue in physiological saline 1:1000. Magnification: ×2,200.

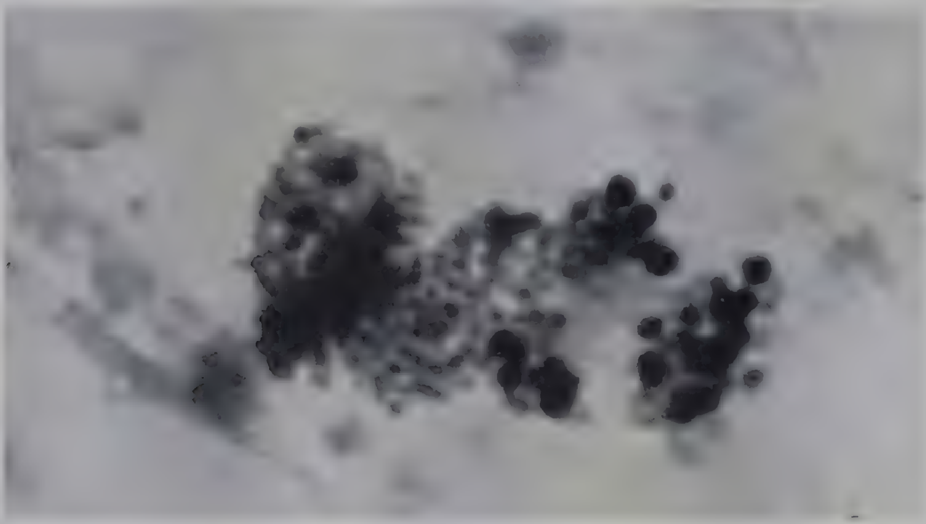

Fig. 2
Mast cell showing partial degranulation after intraperitoneal injection of histamine. Notice the basic structure simulating a sponge or a honeycomb. Staining: toluidine blue in physiological saline 1:1000. Magnification: ×2,200.

Fig. 3
A mast cell during disruption and a macrophage containing some metachromatic granules. Staining: toluidine blue in physiological saline 1:1000. Magnification: ×2,200.

FIG. 4
Mast cells influenced by hydrocortisone acetate. The cells are vacuolated and partly degranulated; the granules are clumped together into irregular masses. Staining: toluidine blue in physiological saline 1:10,000. Magnification: ×1,500.
(*Acta endocrinol.*, **22**, 157, 1956.)

FIG. 5
Two atretic follicles (rabbit ovary) surrounded by mast cells. Staining: toluidine blue in 40 per cent ethyl alcohol 1:1000.

Fig. 6
Dermographic stripe in the skin of a patient suffering from urticaria pigmentosa.

The disruption following upon application of water is probably due to osmosis.

The rapid release of granules from the mast cells after local application of hyaluronidase was interpreted as a compensatory reaction to the topical situation established by a breakdown of the hyaluronic acid of the ground substance, and an increase in the diffusivity of water in the tissues. It is noteworthy that the cells are insignificantly or very slowly altered by the influence of a hyaluronic-acid-containing Tyrode solution. The last-mentioned fluid is possibly the real control fluid affording a physiological environment for the mast cells.

A common denominator of the effects elicited by histamine, histamine-liberators, serotonin, water, physiological saline, Tyrode's solution and hyaluronidase is the increased water content of the environments of the mast cells. Histamine increases capillary permeability, and histamine-liberators are supposed to cause a tissue oedema of a similar kind and genesis.

The implications of degranulation are not fully understood. The present observations, however, indicate that a mast-granule release begins to change free water in the tissues into a mucinous fluid, a sort of connective tissue ground substance. Any disturbance in the physico-chemical balance between the intra- and the extracellular substance may immediately elicit a mast cell response.

The perivascular situation of many mast cells reminds one that they may to some extent be functionally linked to vascular permeability, being ever ready to change a perivascular oedema into mucinous ground substance.

The connective tissue formation caused by carcinoids and their metastases (Hedinger and Gloor, 1954) might possibly be related to and started by the degranulating effect of serotonin, unceasingly released, upon the mast cells. The tissue oedema induced by this agent could be primary to an organization process.

It is my impression that the cortisone group of steroids exerts a cytotoxic effect on the mast cells. It gives rise to other changes besides degranulation. Thyrotrophin and growth hormone appear to stimulate the mucinous system, and the formation of mucopolysaccharide seems to be primary in relation to the binding of water in the tissues.

It is my opinion that the mast cell release of granule substance may be *active* as a consequence of hormonal stimulation, or *passive* as a

consequence of an increased content of free water in the connective tissue ground substance.

II. BASOPHIL LEUKOCYTES AND TISSUE MAST CELLS

The finding by Graham and her associates (1955) that the basophils of the blood contain relatively large amounts of histamine seemed extraordinarily interesting in view of the current discussion as to whether mast cells and basophil leukocytes are the same type of cell or carry out similar functions. Staining of the cytoplasmic granules corresponds to the staining of the granules of the tissue mast cells; they are metachromatic, coarse and of a size like those of the tissue mast cells. The basophil leukocytes are somewhat smaller than the mast cells of connective tissue. The nucleus of a basophil leukocyte is fragmented, usually bisegmented, whereas that of a tissue mast cell is generally unsegmented. The basophils are formed in the bone marrow, whereas the tissue mast cells are believed to be recruited from perivascular mesenchymal cells. Mast cells have never with certainty been observed to pass through vascular walls, but they are at times seen among endothelial cells, and they are nearly always accumulated along the smaller vessels: Do the basophils form mucopolysaccharide, perhaps anticoagulant substances of the heparin type? Is heparin formed mainly by the basophil leukocytes and hyaluronic acid by the tissue mast cells? Do they both release histamine?

The number of basophil leukocytes has been reported to decrease in individuals under the influence of cortisone, like mast cells (Code *et al.*, 1954).

In rabbits' ovaries the following findings have been made: 9–18 hours after copulation or after an injection of gonadotrophic hormone, acting mainly as luteinizing hormone (Physex), Dr. Francis Zachariae and I were able to demonstrate a remarkable phenomenon which we are at present studying in more detail. In the histological sections the blood vessels of the ovaries and tubes were densely packed with basophil leukocytes or mast cells; there were cells with segmented nuclei and others with unsegmented nuclei. In the ovaries, around recently ovulated follicles and around atretic follicles (Fig. 5) there were accumulations of these metachromatic cells. Later they were demonstrable inside the solid masses of cells which subsequently disappeared. Counting of basophil leukocytes in the blood of such

rabbits has so far revealed an abrupt drop and a subsequent, even increase. This is remarkable considering that mast cells are scarcely seen in non-stimulated rabbit ovaries. At the moment Dr. Bouseila of our group is studying the variations in these cells and their relation to the tissue mast cells.

The presence of mast cells or basophils in stimulated ovaries might indicate a task in the repair process following ovulation or in the organization of an oedema. Whether the phenomenon may have any correlation to the presence of hyaluronic acid reported to be present in the cumulus of the egg is an open question.

I would like to supply one further argument in this discussion: a patient with generalized urticaria pigmentosa (Fig. 6). He proved to have up to 10.3 per cent mast cells in the bone marrow (normally less than $\frac{1}{2}$ per thousand), up to 9.5 per cent basophil leukocytes in the blood (normally less than $\frac{1}{2}$ per cent) (Table IV) and in his rather

TABLE IV

White Blood Pictures

	July 1955	July 1955	Sept. 1955	Oct. 1955	Jan. 1956	Feb. 1956	Apr. 1956
W.B.C.	5,600	7,000	7,700	8,000	8,000	10,000	7,600
Differential count, %:							
Rod shapes	2.0	2	1.5	4.5		1.5	2.0
Segmental	42.5	53	62.0	62.0	57	62.0	60.0
Eosinophils	3.5	7	4.5	4.0	6	4.5	2.5
Basophils	*9.5*	*5*	*3.0*	*4.0*	*5*	*3.0*	*7.5*
Lymphocytes	37.0	29	25.0	21.5	30	25.0	20.5
Monocytes	2.5	4	4.0	4.0	2	4.0	7.5

enlarged liver there were enormous quantities of mast cells. In addition, he had disseminated xanthomata. He did not show any signs of heparinaemia, and the heparin-resistance, protamine-titration and thrombin-generation tests were negative. Clotting time, also after rubbing of the skin, was normal. He did not have attacks of flushing, but itching was a predominant symptom. The blood lipid values were within the limits of normal, but the cholesterol values were relatively high.

This case gives some support to the assumption that basophil leukocytes and tissue mast cells are the same type of cell, but it must be pointed out that the case is unique.

GROUP DISCUSSION

DR. SNELLMAN wondered what evidence existed that heparin was a precursor of hyaluronic acid. He felt it was unlikely that such a complicated chemical change would occur *in vivo*. He said that there was no

hyaluronic acid in liver capsule in spite of mast cells. Dr. Asboe-Hansen said that heparin and hyaluronic acid were both acid mucopolysaccharides. The mast cell was the only connective tissue cell known to contain mucopolysaccharide substances. The metachromatic material which, evidently, the mast cell gives off to the ground substance was sensitive to hyaluronidase. The mast cells might contain building stones common to heparin and hyaluronic acid.

The content of hyaluronic acid in the liver capsule might be low even if the mast cell content was high. There was no doubt that these cells had several functions. It should be pointed out also that there were chemical differences between the mast cells of different species and between mast cells in different locations of the same individual. Intraperitoneal injection into mice of radioactive sulphate, followed by autoradiography of experimental tumours containing large amounts of mast cells, showed an uptake in most, but certainly not in all, mast cells. There were also differences in PAS reactivity and lipid staining.

Dr. Meyer spoke of recent work chemically desulphating all polysaccharides including heparin. Such desulphated heparin still retained positive rotation, and was resistant to both types of hyaluronidases. Work on the chemical structure of heparin showed that it contained a repeating disaccharide which was not the same as the disaccharide of hyaluronic acid. In tissue culture of rat subcutaneous tissue, rat and human skin, membranous and long bone and synovial tissue, the fibroblasts also produced hyaluronic acid. He could not see how one could equivocate the mast cell with the fibroblast.

Dr. Asboe-Hansen could not be persuaded that the problem could be solved by tissue culture experiments. One might have granulated mast cells and even non-granulated ones; the last possibility must certainly be taken into consideration in tissue cultures and in mesenchymal tumours.

Dr. D. S. Jackson mentioned work done by Dr. Robertson in Vermont on the production of granulomata in Vitamin C deficient guinea-pigs. Little or no sulphated polysaccharides were produced but fairly large amounts of hyaluronic acid were found. In this situation there were no obvious mast cells but large numbers of fibroblasts.

Dr. Asboe-Hansen said that in fact, the proper identification of mast cells involved ideal fixation, preparation and histochemistry, and perhaps isotope studies as well.

Dr. Meyer pointed out the wide variety of unrelated polysaccharides. The mast cell only contains one type of polysaccharide, together with a monosulphated ester which may or may not be a precursor of heparin. Human aorta contained few mast cells but a lot of this monosulphated ester. No one had yet isolated a sulphated mucopolysaccharide from synovial fluid, although there was a very small quantity of sulphate. The only

polysaccharide isolated from synovial fluid was hyaluronic acid, which of course, is not sulphated.

Dr. Asboe-Hansen said that mast cells may contain several polysaccharides in several stages of polymerization and sulphation. Synovial membrane has been found to contain a lot of degranulated mast cells near the cavity and heavily granulated ones deeper down. The metachromasia of the amorphous ground substance varied inversely with the intracellular metachromasia.

In answer to several points raised by Dr. Neuberger, Dr. Asboe-Hansen said the life span of mast cells was unknown. Mitoses were scanty even in very large accumulations of mast cells seen in experimental tumours in mice. The mast cells appeared to regenerate from the perivascular mesenchyme.

Several participants thought autoradiography might prove informative on this point, but Dr. Gillman suggested that the mast cells need not necessarily all be in the same stage of physiological granulation or metabolism and consequently the cells may differ in uptake of isotopes. He felt that Dr. Asboe-Hansen's illustrated accumulations of mast cells in ovarian sections may not have been derived from the blood, but may perhaps have arisen locally from the ovarian connective tissue phagocytes. In support of this view he projected two colour slides from calciferol-treated rats in which the gastric muscularis, forty days after calcification, contained dense collections of mast cells around the previously calcified necrotic areas. Dr. Gillman considered the sequence of events was possibly as follows: (*a*) degeneration or necrosis of tissue (for example, following mineralization with calcium); (*b*) accumulation of metachromatic staining material in and/or around, or perhaps even derived from this necrotic material; (*c*) the appearance, one or two days later, of large numbers of cells which phagocytized this metachromatic debris, thus coming to look like mast cells. Thus, he did not consider that this metachromatic material, regularly encountered in such necrotic areas, was necessarily derived from the mast cells. In fact the 'mast cells' in these circumstances were perhaps phagocytes loaded with ingested metachromatic material, suggesting that one type of mast cell may arise in this way.

Dr. Baló asked if there were any experiments on the phagocytosis of heparin. He recalled that Walton, in 1954, claimed heparin could not be phagocytized, although other workers, Asplund, Borrel and Holmgren, found that small amounts of injected heparin could be taken up. Walton believed this previous work was due to contamination of the heparin used with other mucopolysaccharides. Jancso, working in his own country on heparin, showed it could be taken up by the cells of the reticulo-endothelial system when injected into connective tissue.

Dr. Asboe-Hansen agreed that, in some instances, it might be difficult to differentiate between macrophages and mast cells but he believed it was possible in living tissues because of the background structure of the mast cells. After cells were disrupted, metachromatic granules were found in both the ground substance and the cytoplasm of the phagocytes. After provoking degranulation of the mast cell, a metachromatic amorphous material appeared around the altered mast cells, except in hyaluronidase-treated connective tissue. In fixed tissue, mucopolysaccharide substances phagocytized by macrophages do not usually appear granular, but as diffuse cytoplasmic metachromasia.

ON THE TOPOGRAPHICAL CYTOCHEMISTRY OF TISSUE MAST CELLS

B. Sylvén

Since the time of Ehrlich it has been widely accepted that the mast cell granules present metachromasia and also show basophilia to basic aniline dyes. This contention further strengthened by the results of Jorpes (1935), Holmgren and Wilander (1937) and Wilander (1938) led to the opinion that the mast cell granules *per se* contain and even 'are' heparin as stated by Jorpes (1946). Subsequently more detailed investigations on the cytoplasmic constituents of tissue mast cells by means of the freeze-vacuum dehydration technique and carefully controlled differential centrifugation methods led to the view that the native heparin material was located most likely as part of the microsomal substance within the intergranular compartment, thus surrounding the basophilic large granules.

Since I am mainly responsible for the latter interpretation you may allow me to discuss and evaluate the evidence in favour of these interpretations, and also to try to reconcile the various views with more recent data on the ultrastructure of mast cell cytoplasm. Let me first say that this question is no great matter of dispute, rather a question of academic interest. It is a matter of small distances, about 100 or a few hundred Å units, which, however, may be of some importance when function and metabolism are placed on more secure grounds. The sub-miscroscopic dimensions of mast cell components would then seem suitable for cell fractionation studies combined with chemical and staining assays of fractions obtained.

The old contention is based on a large accumulation of staining results routinely performed on tissues pretreated with various fixatives. In tissue sections of a thickness between 5 to 10 or more microns, large mast granules are seen, having a diameter in general between 200 to 500 millimicrons. The large granules were found to exhibit different degrees of metachromasia, some apparently red stained, others in tones of bluish-red, distinctly violet, and bluish or bluish-black. Very little intergranular cytoplasm was seen, and

those who have described this material by using other staining methods quite strictly state that 'the intergranular cytoplasm is clear and homogeneous (*oxyphilic*!)' (Michels, 1928, cit. from p. 258). Little attention was paid to other staining methods. It would be worth while to mention that the granules generally take up hematoxylin and that some large granules appear to be stained also with eosin and orange G (Lehner, 1924).

The granules further show a higher degree of refractivity than the rest of the cytoplasm, presumably due to a larger mass content, which facilitates their identification in unstained samples. A number of more specific staining reactions suggest that the granules may contain glycogen, protein, lipid, possibly RNP and some further Schiff-positive carbohydrate(s) or lipid material and a variety of enzymes.

The staining results would thus seem to suggest that the mast granules contain a number of different non-classified components. Their marked basophilia has to be explained by the presence of some acid material, which does not seem to be heparin but possibly nucleic acid or some acid polysaccharide. So far, however, the intergranular cytoplasmic materials have not been considered in detail.

At this point I would like to mention the position of the problem around 1940 in the laboratory of Hjalmar Holmgren. Together with Jorpes and Wilander he found evidence that mast cells contained heparin and was studying the metachromasia of mast cell granules, their morphology, histogenesis, etc. However, he was worried about the variations in staining characteristics noted in many mast cells; some granules were not metachromatic but instead strongly basophilic and stained blue to bluish-black. This was then believed to signify different functional stages, i.e. suggesting that some granules in the course of heparin production had not yet fulfilled the complete synthesis. Holmgren was quite convinced that the granules were not made up of pure heparin, but believed that heparin was a fair part of the granular mass. Incidentally, little attention was then paid to the new approaches in cytochemistry developed by R. R. Bensley, N. L. Hoerr, A. Claude and others. At that time, we almost forgot the possibility that heparin might be located in the seldom observed amorphous intergranular substance.

Now, my interest was aroused by histological studies of mast cells. Most aqueous and other fixatives recommended for the effective precipitation of metachromatic material were found to involve

shrinkage, dissolution, swelling or other artifacts hampering detailed analysis. Therefore, investigations on fresh-frozen and vacuum-dehydrated tissues were found more reliable as far as the microscopy of water-soluble acid polysaccharides of connective tissue and mast cells were concerned. In thin fresh-frozen and dried sections purely basophilic and non-metachromatic mast cell granules were often seen. Pictures of mast cells, like those published (Julén, Snellman and Sylvén, 1950), indicate that the 'amorphous' intergranular substance (cf. Sylvén, 1950) stained *metachromatic and oxyphilic*. Great caution was further justified in interpreting the staining reactions of cytoplasmic granules with dimensions below or very close to the lateral and axial resolving powers of the light microscope. Other observations suggested that metachromatic material was easily dissolved or could be released during physiological conditions, leaving strongly basophilic granules to be seen. Cramer and Simpson (1944) even described 'ametachromatic' mast cells appearing in the course of carcinogenesis experiments.

It then seemed necessary to apply chemical fractionation methods in order to obtain more detailed information on the cytochemistry of these cells. At this stage, co-operation was obtained with Dr. O. Snellman of the Institute of Biochemistry. Only the main findings of immediate interest to the topical problems of heparin localization will be outlined below.

First, a mild method suitable for the extraction of the native heparin complex was found by using thiocyanate solutions, which present a unique capacity to bring the metachromatic mast cell material almost quantitatively into suspension. This material was dissolved without changing the morphology or basophilia of the large granules, and it was therefore probably not bound to the granules or the possible union was by very weak bonds only.

The fractional separation of mast cell components was accomplished according to general procedures (Julén, Snellman and Sylvén, 1950) using isotonic phosphate buffer. A very strict pH control was found necessary in order to avoid damage to the large granules and still more to avoid aggregation and precipitation of the microsomal material. Claude (1946) described how the large liver granules aggregate and later disintegrate in more acid media. We found that the microsomal mast cell material disperses only around pH 7, below pH 6 there is an increasing tendency to flocculation. An increase in acidity is thus most deleterious since the microsomal

material precipitates on the surface of the large granules and becomes also very firmly aggregated to all sorts of other cell materials in suspension. This was a real catch in the fractionation technique and seemed further to explain part of the artifacts produced by the histological preparation methods (cf. below).

If now the pH was kept at 7 during the whole fractionation procedure we obtained (*a*) a large granules concentrate to be further washed and cleared, (*b*) a microsomal fraction, and (*c*) a final supernate. Neither of these fractions have been claimed to be quite 'pure' (cf. Novikoff *et al.*, 1952). The first large granules concentrate contained also some microsomal material, and the supernate contained some very fine particles which could not be sedimented by centrifugation at 60,000 g. The bulk of heparin material was part of the supernate as a sub-microscopic particulate matter with diameters below 10 millimicrons. The final supernate contained four different non-sedimentable fractions distinguished by electrophoretic mobility, and only one of these fractions (called u_6) contained heparin (Snellman, Sylvén and Julén, 1951). This native heparin material was found to be a heparin-lipo-protein complex with a particle size of about 100,000 and an electrophoretic mobility of 5.9×10^{-5} sq. cm./volt/sec. Purified heparin shows under similar conditions a particle size of about 17,000 and a mobility of $18 - 22 \times 10^{-5}$ sq. cm/volt/sec.

All fractions obtained during the course of fractionation and all washings were analysed. *The only fractions showing metachromasia were those which contained heparin*, mainly the final supernate. *Washed large granules were free of heparin.*

The sub-microscopic particulate matter in the final supernate as well as part of the sedimentable microsome mass showed a marked *oxyphilia*, which we ascribe to the presence of basic protein, contained in any one of the two or three additional protein components devoid of heparin. The oxyphilia was of the same order as that seen in freeze-vacuum dehydrated mast cell cytoplasm. Both the sedimentable and non-sedimentable microsomal material showed a similar colloidal behaviour to various hydrogen ion concentrations as other microsomes have done. Further, neither the large granules, nor the microsomes presented a very marked solubility in the native state as long as the pH was kept at 7. Heparin was not dissolved from the native complex even when the suspensions were kept for days.

Later on, Hedbom and Snellman (1955) isolated and analysed the

washed non-metachromatic large granules concentrate. Electron micrographs showed well-preserved large mast granules with diameters between 200 to 500 millimicrons. Ultra-thin sections have not yet been made. The general composition of the granules was: protein 72 per cent, lipids 24 per cent, RNA 3.6 per cent and some small amounts of carbohydrates and histamine. Some enzyme activities were demonstrated. The amount of heparin was less than 0.3 microg. per gramme of washed large granules. *Practically no trace of heparin was thus found*; traces of hexuronic acid were probably present. Thus, the composition conforms well with the general composition of other mitochondria and large secretory granules.

Our joint results led to the suggestion that the native heparin complex was very likely housed in the intergranular cytoplasm of mast cells as part of the microsomal material. A tentative reconstruction of the possible sub-microscopic organization of mast cell cytoplasm was made on this basis, and it was emphasized that our interpretation would explain the variable metachromasia of mast granules. With reference to the marked basophilia of mast granules we may point to the high content of RNA and/or other protein or lipid components, but we did not find a large amount of acid polysaccharide material as had been expected. Whether some smallish amount of a carbohydrate heparin precursor or other ground substance polysaccharides is present in the granules remains to be investigated. The granules appear to contain a trace of a non-metachromatic polysaccharide material devoid of anti-clotting effects but its nature has not yet been investigated. Thus from chemical and structural points of view it seems to us very unlikely that heparin is a precursor to hyaluronic acid, or that the latter is convertible into heparin.

We have so far no topical knowledge of the enzyme distribution and cannot suggest where the different steps of heparin synthesis may take place. To our mind it seems, however, plausible that the synthesis occurs in the ground substance of the cytoplasm in close co-operation with the large granules, perhaps in such a way that the final esterification is performed near the surface of the large granules.

Our findings and interpretations have led to different comments and a certain amount of confusion is noted in later reports (Zollinger, 1950; Friberg, Graf and Åberg, 1951; Wislocki and Fawcett, 1951; Montagna, Eisen and Goldman, 1954). However, it does not seem that the usual histological techniques, phase-contrast studies and the

inspection of thick whole-mounts of mast cells have progressed sufficiently to allow definite conclusions to be drawn from studies undertaken at the limit of or below the resolving power of the light microscope. Moreover, the use of acid solutions as fixatives (Helly's fluid – pH about 3.3 – used by Montagna *et al.*, 1954) and/or during staining will undoubtedly precipitate the mast cell intergranular microsome material in the same way as in *in vitro* experiments. The intergranular material following denaturation will unfortunately precipitate and stick to the surface of the large granules as well as to other elements, which thus will acquire a metachromatic shade.

From phase-contrast studies, Zollinger (1950) claimed that the granules had a water-insoluble protein skeleton and somehow inside this the metachromatic heparin component would be harboured. It is, however, difficult to accept this speculation when the granules so easily can be prepared in an apparently undamaged state (cf. Hedbom and Snellman, 1955) and are shown to be almost devoid of heparin (cf. above).

Köksal (1953) claimed to have obtained some metachromatic mast granules by shaking tissue spreads in distilled water for 1–2 hours without pH control. It seems clear that he obtained mast granules surrounded by a coating of metachromatic material. On the contrary, Zollinger found that mast cell granules treated with water released their metachromatic material, became orthochromatic and then lysed.

Some investigators have expressed some doubt as to our observation that the supernate fraction, containing – among other things – the native heparin complex, is oxyphilic and not basophilic. This may, however, be explained by the presence of oxyphilic protein material together with the native heparin complex. It seems quite evident from osmotic and other considerations that heparin in the native state cannot exist as a dissociated polyelectrolyte. We have said that it seems to be combined with a lipoprotein moiety, which may stain with eosin, and that there are other microsomal components of cytoplasm which may show eosinophilia. The heparin complex as such has enough electronegative surface charges available for showing metachromasia (cf. Sylvén, 1955).

Another question has to be considered if in our fractionation experiments we overlooked a dissolution effect leading to a very rapid and early release of heparin when the tissue mast cells are suspended in phosphate buffer. This would be an objection common

to differential fractionation procedures as such performed in aqueous solutions (cf. Novikoff *et al.*, 1952). Generally speaking, if a water-soluble substance forms part of any granular fraction, some of it may be found in the supernate. But as far as we know, no one has attributed a substance solely occurring in the supernate and not at all recovered in the granular fraction as belonging to the granules. Thus, our mast cell fractionation results seem only explainable on the assumption that the native heparin complex occurs outside the granular membranes.

This is as far as present evidence goes. We all hope that further details of the fundamental cytochemistry of mast cells will be revealed leading to a closer understanding of the physiology of these peculiar cells. The important discovery of histamine in mast cells (Riley and West, 1953), recently corroborated by chemical extractions (Hedbom and Snellman, 1955), lends further support to the assumption that connective tissue mast cells take part in local tissue reactions of various kinds.

GROUP DISCUSSION

Dr. Neuberger said that the only possible criticism against Dr. Sylvén's interpretation of his data was the very remote possibility of loss of heparin from the granules during his isolation process. If the molecular weight of the native heparin-protein complex were high, this possibility would be unlikely. If it were only 15,000 or less, it might easily leak out.

Dr. Sylvén replied that the isolated heparin particle size was about 17,000; but under similar experimental conditions the native heparin-protein complex had a particle size (molecular weight) of about 100,000.

Dr. Snellman felt that the electron microscopic pictures showing the granules as undamaged round balls would make it highly improbable that the heparin complex had leaked out from the granules during the isolation processes.

Dr. Gillman suggested that heparin may be intimately connected with the minerals and their movements (especially calcium) in the mast cells and elsewhere. Heparin also seems to exert some lipaemia clearing role not only in the circulation but also *in vitro*. The presence of calcium ions seems to be necessary for the breaking of the chylomicron emulsion with the formation of a creamy layer of agglutinated chylomicrons that appears above the cleared lipaemic plasma and serum after centrifugation. Also, mast cells accept a certain amount of silver staining with von Kossa

method, indicating a moderately high mineral content in these cells. This heparin-calcium or -mineral relationship would appear to be of physiological significance. For example, prolonged repeated injections of heparin or of sulphated mammalian hyaluronic acid into rabbits, kept on a high fat diet, frequently produced spontaneous fractures in various bones, especially the vertebrae and humerus, indicating changes in osseous minerals perhaps induced by these mucopolysaccharides binding with leaking-out osseous minerals.

Both Dr. Gross and Dr. Gillman agreed that histologists and chemists working with tissue fractions should co-operate in assessing unavoidable artifacts in both types of investigation, such as trauma to mast cells following pressure by cover slips, or by the development of ice crystals, even in frozen-dried tissue, or possible damage to cells during high-speed centrifugation.

Dr. Sylvén further explained that variations in the coating of the mast cell granules with microsomal material most likely would explain their variable staining reactions as to metachromasia and basophilia; in some instances the granules are purely basophilic.

Dr. Meyer said that the low electrophoretic mobility seems surprising in view of the high charge of heparin and the apparent dissociation of the native complex as evidenced from the binding of toluidine blue.

OBSERVATIONS ON THE PRESENCE AND METABOLISM OF PLASMA PROTEINS IN SKIN AND TENDON

A. Neuberger

In an earlier paper (Harkness, Marko, Muir and Neuberger, 1954) a protein fraction was isolated from the skin of rabbits which in solubility and electrophoretic behaviour closely resembled serum proteins. Further information on this fraction was desired, partly as a contribution to our knowledge of the protein composition of connective tissue, but also because a more complete identification of the proteins present in this fraction might have a bearing on the general metabolism of plasma proteins. Isotope studies by various authors (e.g. Miller, Bale, Yuile, Masters, Tishkoff and Whipple, 1949; Wasserman and Mayerson, 1951; Myant, 1952; Cohen, Holloway, Matthews and McFarlane, 1956) have produced fairly conclusive evidence that at least half, and probably more, of the total plasma protein present in the body is outside the circulation. Apart from the information obtained by Coons's fluorescent antibody technique (Gitlin, Landing and Whipple, 1953) there is little precise knowledge as to where exactly in the body this plasma protein is situated.

The purpose of the experiments to be described was as follows. In the first place it was desired to have an unequivocal identification of the proteins of this fraction. Secondly, we wished to get a reliable estimate of the quantity of the various proteins and thirdly, we wanted to study the dynamics of the exchange of the plasma protein present in connective tissue with the plasma protein in the plasma. Finally, it was desired to extend the investigation to tissues other than rabbit skin. The experiments to be described were carried out jointly with Dr. D. J. Perkins and Dr. J. H. Humphrey at the National Institute of Medical Research at Mill Hill and at St. Mary's Hospital Medical School.

GENERAL PLAN OF EXPERIMENTS

The tissues studied were rat-tail tendon, rabbit tendon, cow tendon and rat and rabbit skin. The tissues were finely ground and then

extracted at a temperature of 0-4° C. three times with 0.15 M. phosphate buffer pH 7.8-8.0. The material was then dialysed and dried from the frozen state. The albumin fraction which appeared to be electrophoretically pure to the extent of 95 per cent was obtained by salt fractionation. Identification of the albumin was done by the following methods, (*a*) electrophoresis, (*b*) immunological behaviour, (*c*) optical rotation before and after denaturation with guanidine, and (*d*) ultraviolet absorption in acid and alkaline solutions. The following proteins were used for injection: (*a*) iodine-labelled albumin and iodine-labelled plasma protein prepared by the method of McFarlane (1956), (*b*) various ^{14}C-labelled albumins, (*c*) ^{14}C-labelled antibody, and (*d*) horse serum albumin. In one experiment radioactive glycine was injected into a rabbit and the serum proteins and soluble skin proteins were fractionated and their radioactivities were measured.

RESULTS

Tendon. The tendon of an adult animal contains relatively few cells and a large part of the total space can be assumed to be extracellular. In the rat-tail tendon only a small amount of protein, 0.1 per cent of fresh weight of the tissue was obtained by extraction with phosphate. With an anti-rat serum prepared in a rabbit a positive but weak reaction to this soluble tendon protein fraction was given. Extraction of cow tendon gave a larger soluble fraction which in free electrophoresis behaved like plasma protein. It gave all the peaks in approximately similar proportions as were found in a sample prepared from plasma, but there was in addition a small fast peak which moved in front of the albumin. A similar, fast peak was observed in the earlier experiments of Harkness *et al.* (1954) with the soluble rabbit skin material. Immunological analysis gave evidence of the presence of all serum proteins. This soluble protein fraction amounted to 0.25 per cent of the fresh weight of the ox Achilles tendon used.

A rabbit was injected with iodine-labelled albumin and the animal was killed 10 days later. There was a relatively large radioactivity per mg. of nitrogen in the phosphate extracts, but the specific activity (counts per mg. of nitrogen) was appreciably lower than for proteins obtained from plasma. This suggests that either equilibration was not complete, or that this fraction contained a considerable

amount of material, possibly 50 per cent, which was not serum protein. A large amount of nitrogen could be brought into solution by extraction with normal alkali. This material had, however, negligible radioactivity suggesting that the protein which is not removed by three extractions with phosphate is not serum protein. Examination of the fractions obtained from rabbit tendon by electrophoresis showed a clean albumin peak, a somewhat less clean γ-globulin peak, whilst the α- and β-globulin fractions were apparently contaminated with appreciable amounts of other proteins.

SKIN

General properties of the proteins. As judged from electrophoretic analysis all protein fractions of plasma were present but there was evidence that the extract contained appreciable amounts of protein other than plasma proteins. The albumin was purified as described; the optical rotations of the native protein in M/15 phosphate and in 3M guanidine hydrochloride were identical for the albumin obtained from skin and from plasma, the values being -52.3° and -52.3° before denaturation and -84.1° and -83.9° after denaturation respectively. The ultraviolet absorption curves both of the serum albumin and the skin albumin were closely similar, but the material obtained from skin seemed to contain material with an unspecific absorption which increased the extinction values over the whole range to a very slight extent. Immunological analysis clearly suggested that a large part of the skin albumin fraction was indistinguishable from the albumin obtained from plasma. It should also be added that the change of rotation which is probably a measure of the rate of denaturation and which occurred on addition of guanidine hydrochloride to phosphate solutions of the skin and plasma albumins was practically instantaneous. It is concluded that, judging by the tests employed, a large part and probably most of the albumin obtained from skin is either indistinguishable from, or identical with, serum albumin.

The possibility had to be excluded that the albumin extracted from skin was mainly derived from the blood present in the blood vessels of the skin. This was done in two ways. Iodine-labelled albumin was prepared and injected into a rabbit. The animal was killed after five minutes and the radioactivity of the phosphate extracts was measured. The radioactivity of the plasma was measured at the

same time. Comparison of the specific radioactivities (in terms of nitrogen) showed that plasma in skin amounted to less than 1 ml. per 100 gm. skin. If we assume that after five minutes the iodine-labelled albumin had completely mixed with the intravascular albumin, but had not penetrated to a significant extent into the extravascular space, it can be concluded that the amount of blood present per 100 gm. skin is less than 1.5 ml. Haemoglobin estimation of the skin sample indicated that the amount of blood was of the order of 0.4 ml. per 100 gm. Thus the two methods, whilst not giving good agreement, suggest that the blood present in skin cannot be more than 1.5 ml. per 100 gm. The results to be discussed later showed that the total amount of albumin which can be isolated from skin is at least 10 or 15 times greater than the maximum amount which can be present within the blood vessels.

Rate of equilibration between plasma and skin. Iodine-labelled plasma proteins were injected into two rabbits and one of the animals was killed after 4 days, while the other was killed after 10 days. The radioactivity in the plasma was measured and also that of successive extracts of skin. When the radioactivities were compared on a nitrogen basis, successive extracts of the four-day experiments showed that the skin extracts had between 23 and 32 per cent of the plasma activities. In the ten-day experiment the average value of five skin extracts was 50 per cent of that of plasma, again expressed on a nitrogen basis. It appeared possible that these differences were due to a variation between animals and a series of experiments was therefore done in which iodine-labelled albumin, carbon-labelled albumin, carbon-labelled antibody and a foreign protein, horse serum, were injected in various combinations into ten rabbits. The experiments showed the following. With iodine-labelled albumin the highest values were found in the 8-16 day experiments where the radioactivity in the skin was equivalent to about 12 ml. of serum per 100 gm. of skin, and the lowest values were observed in the one experiment when the animal was killed 8 hours after injection. The values between 2 and 8 days varied somewhat, but were equivalent to figures ranging between 4 and 8 ml. of serum per 100 gm. of skin (Fig. 1). It appears that a steady state, which possibly indicates complete equilibration between plasma and skin, is reached in about 8 days. It seems to require about 3-4 days to get about half the skin albumin replaced by plasma albumin. But in view of the marked variation between animals these values are only approximate. In

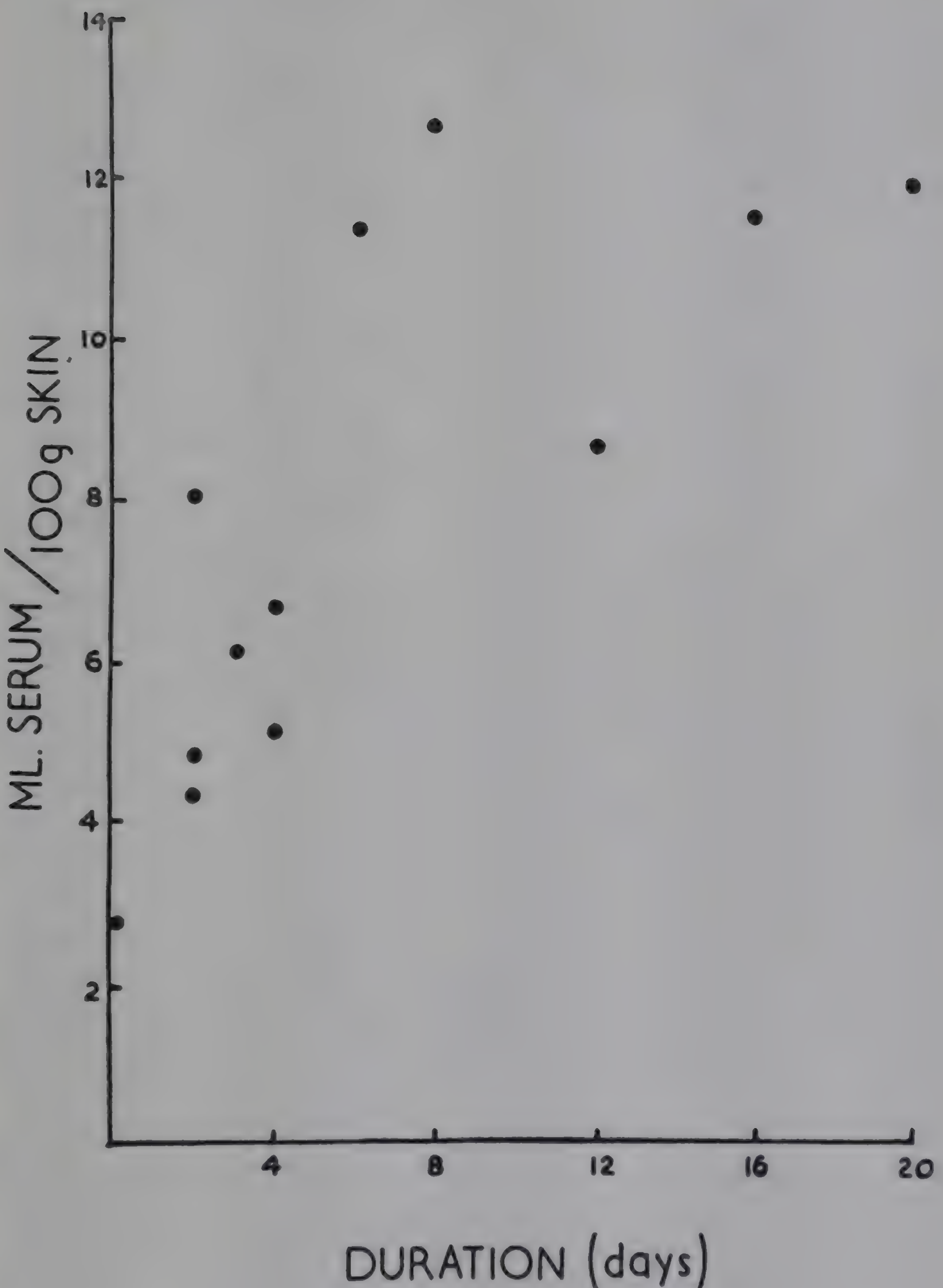

FIG. 1

The changes of radioactivity of skin albumin with time

The radioactivity of skin extracts were compared on a nitrogen basis with that of plasma albumin obtained from plasma at the same time. The results were calculated in terms of ml. of serum per 100 gm. skin.

one experiment carbon labelling and iodine labelling gave similar results suggesting that the latter method is not likely to yield misleading information.

Heterogeneity of the skin albumin fraction. The experiments discussed above indicated that either complete equilibration between the skin and plasma is very slow or that the fraction which is designated albumin consists not only of serum albumin but also contains another protein, possibly formed in the skin, having a solubility characteristic of albumin. [^{14}C] Glycine was therefore injected into a rabbit and the animal was killed 8 hours after injection. The albumin fraction from skin was fractionated by column electrophoresis (Porath, 1956). Serum obtained from this animal was also subjected to fractionation by the same technique. Skin extracts gave a fraction designated I with a specific activity of 414 counts/min./mg. carbon. This material was examined serologically with an anti-rabbit albumin serum obtained from a goat. Quantitative precipitative reaction was carried out both on Fraction I of skin extract and on two preparations of rabbit serum albumin. Fraction I had about 87 per cent of the precipitating power of that of authentic serum albumin indicating that it consisted of approximately 87 per cent pure albumin. A second fraction obtained by column electrophoresis of the skin extract designated Fraction II, which was smaller in quantity than Fraction I, had a radioactivity corresponding to 597 counts/min./mg. carbon. Serological analysis indicated that it was immunologically at least not equivalent to Fraction I. It contained some material capable of reacting or cross-reacting with anti-rabbit albumin, as well as material capable of reacting with some other antibody in the antiserum. Fraction II contained less than a third of material behaving serologically like albumin. There was a third fraction obtained which was not examined serologically which had a still higher radioactivity, i.e. 957 counts/min./mg. It was therefore concluded that the skin extract which represented an albumin fraction as defined by solubility and electrophoresis contained material amounting to probably about 60 per cent of the total protein, which was immunologically identical with serum albumin but it also contained at least two other components. The high radioactivity of the fractions containing predominantly material other than serum albumin is compatible with the assumption, but does not prove that this other protein is made in the skin from the radioactive glycine supplied.

DISCUSSION

A large proportion, probably about 95 per cent of that part of the water-soluble protein fraction of skin which is not precipitated by 26 per cent sodium sulphate, resembles in its electrophoretic behaviour and general properties plasma serum albumin, but the fact that its specific activity, in the experiments in which iodine-labelled albumin was injected, never exceeded 65 per cent of that found at the same time in the circulating albumin indicates that the skin fraction designated as albumin was not pure. Indeed the results of the fractionation with the Porath column showed that this material contained at least two fractions, one closely resembling serum albumin and another fraction which may itself be heterogeneous and which consisted probably mainly of a protein of albumin character, possibly produced locally by cells in the skin. Our data do not allow an accurate estimation to be made of the relative proportion of serum albumin in the albumin fraction obtained from skin, but the isotope and immunological data suggest that serum albumin may constitute about 60 per cent of the total albumin.

The claim of identity of this material with plasma serum albumin is based on its serological behaviour, electrophoretic mobility, its optical rotation under two sets of conditions and finally its ultraviolet absorption spectrum in acid and alkali. The chemical and physical criteria used are by no means conclusive, especially since the amino-acid composition of the various fractions was not determined, but the immunological criterion is believed to be quite decisive. In addition, the results of the various isotope experiments indicate beyond any reasonable doubt that a large part of the soluble protein consists of serum albumin.

The evidence with regard to the other plasma protein fractions is less conclusive. Electrophoretic evidence has been obtained suggesting the presence of all three globulin fractions but in most of our experiments peaks were not as clearly defined as might be desired and there were strong indications of the presence of significant amounts of other proteins. There is thus little doubt that skin contains serum globulins in addition to serum albumin, but possibly not in the same proportions as in the blood.

Total amount of serum protein in skin. The data represented showed a considerable scatter between different animals and this and the lack of complete purity of our fractions make it impossible to produce a

very accurate estimate of the amount of albumin present in rabbit skin. But the data, particularly the results obtained by the use of iodine-labelled albumin, suggest that 100 gm. of skin may contain plasma protein equivalent to that present in approximately 12-16 ml. of plasma. This is equivalent to a protein content of about 1 gm. of plasma protein per 100 gm. of skin. If it is assumed that about 60 per cent of the total space of skin is extracellular, and plasma proteins are confined to the extracellular space, this would mean that the concentration of plasma proteins in the extracellular compartment of skin is about 1.5 to 2 per cent. This value is similar to that obtained for plasma protein in lymph from the limbs and thus is likely to be a reasonable estimate. On the basis of this rather rough estimate it would appear likely that the total amount of plasma protein in the skin is equivalent in amount to about 30 per cent of that present in the circulating blood. Since the total plasma protein is about 2.25 or 2.5 times greater than the plasma protein in circulating plasma, it is tentatively concluded that the skin accounts for about 25 per cent of the total extravascular plasma protein.

Dynamics of the exchange between plasma and skin. The results shown in Fig. 1 and other experiments not described in detail, in which ^{14}C-labelled proteins, labelled antibody and horse serum were used, indicate that a steady state of the relative activity of skin serum proteins as compared with circulating serum is reached about 8 days after injection. This steady state probably means that complete equilibration is being approached. The differences between animals are too great to permit a mathematical analysis of the data of the type discussed recently by Campbell, Cuthbertson, Mathews and McFarlane (1956) and we do not know whether the exchange reaction can be described by one first-order reaction. In other words we do not know whether skin is homogenous with respect to exchange of plasma protein. If the reaction were simple and of first order, the 'half-life' of skin serum protein with regard to exchange with plasma, is likely to be about 4 days. These very rough calculations apply mainly to albumin in skin, and no estimates can yet be given for the globulins of skin or for plasma proteins in other types of connective tissue.

SUMMARY

The presence of plasma protein has been demonstrated qualitatively by electrophoresis and serologically in tendon and in skin.

The albumin fraction has been isolated in a partially purified form and shown to have similar properties as the albumin isolated from serum. The amount of plasma protein in rabbit skin is calculated to be about 1 gm. per 100 gm. skin, and to account for about 25 per cent of the total extravascular plasma protein. The rate of exchange between the plasma protein, particularly the albumin between skin and plasma has been measured by using suitably labelled proteins. Results indicate that equilibration may be approached in about 6–8 days.

GROUP DISCUSSION

Dr. Grassmann said that very small amounts of the component moving faster than albumin on electrophoresis ('pre-albumin') were present also in plasma and that larger amounts could be detected in extra-vascular fluids such as cerebrospinal fluid.

In reply to a question from Dr. Grassmann, Dr. Neuberger stated that the albumin isolated from skin was identical with plasma albumin in its ultraviolet spectrum and tyrosine and tryptophan contents but that a complete amino-acid analysis had not yet been done.

In reply to Dr. Snellman, Dr. Neuberger stated that he had no evidence that iodination affects the properties of albumin so long as the iodination is only slight and is carried out under the conditions given by MacFarlane in the *Biochemical Journal*.

Dr. Gross referred to the work of Gitlin who used the fluorescein-labelled antibody method of Coons which indicated that fibrinogen, some of the globulins and possibly albumin occurs inside the cells, possibly in association with lipids.

He also referred to work of Boas who found approximately 4 per cent plasma proteins in the subcutaneous tissue of the rat, even though there is a negligible quantity of blood.

Dr. Bowes asked if the complete removal of activity on extraction with phosphate buffer referred to both the ^{14}C-labelled glycine and the iodinated protein or just to the iodinated protein.

Dr. Neuberger stated that no work had been done on the extraction of skin after labelled glycine had been given because some of the proteins synthesized in the cells of the skin were insoluble in the buffer. After complete extraction with phosphate buffer, only a very small amount of the iodinated protein remained in the skin residue.

Dr. D. S. Jackson stated that in a neutral salt extract of the granuloma produced by injection of carageenin he had found an albumin not quite

identical with plasma albumin and suggested that it was similar to the albumin found in subcutaneous tissue by Dr. Neuberger.

Dr. Orekhovitch said that, for solving the problem of the identity of the blood plasma proteins with some of the skin proteins, a complex method should be applied, depending on a combination of paper electrophoresis or paper chromatography with immunobiological methods. This complex method was worked out by Gurvick, and a partial description of it was published in *Clinica Chimica Acta*.

STUDIES ON THE FIBROGENESIS OF COLLAGEN. SOME PROPERTIES OF NEUTRAL EXTRACTS OF CONNECTIVE TISSUE[1]

JEROME GROSS[2]

Revelation of the mechanism of fibrogenesis has seemed tantalizingly close during the past three decades primarily through morphological studies including both light and electron microscopy, of embryonic growth, wound healing and tissue cultures. However, the process and site of polymerization of collagen molecules to fibrils are still undisclosed.

One approach to this problem has been made via the study of the behaviour of collagen in solution particularly its reconstitution or precipitation in the form of characteristically striated fibrils as visualized by electron microscopy (Schmitt *et al.*, 1942; Bahr, 1950; Noda and Wyckoff, 1951; Gross *et al.*, 1952; Vanamee and Porter, 1951). Schmitt, Gross and Highberger (1955a, b) have characterized what they believe to be the fundamental building block of the fibril called by them 'tropocollagen' (Gross *et al.*, 1954). As deduced from electron microscopic studies on the precipitation and interconversion of several different 'long spacing' and native forms of collagen from solution (Highberger *et al.*, 1951; Schmitt *et al.*, 1953; Gross *et al.*, 1954), this particle is a rigid rod of the order of 2000–3000 Å long and less than 50 Å wide. These observations were made on collagen derived from three different mammalian species and one fish (carp swim bladder tunic from which ichthyocol is derived). Physical chemical studies on ichthyocol by Boedtker and Doty (1955, 1956) confirmed the existence of such a structure in acid citrate solutions at low concentrations. Their measurements on dilute solutions indicate the presence of a remarkably homogeneous population of relatively rigid rod-shaped particles, 2900 Å long by 14 Å wide, with a molecular weight of 340,000. Hall (1956) has

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[2] Established Investigator of the American Heart Association, Inc., New York.

been able to visualize the tropocollagen particles directly by electron microscopy and has arrived at a mean length of 2200 Å and a width of about 15 Å. It is thought at this time that the tropocollagen particle is the smallest unit which can be directly built into a characteristic collagen fibril.

Earlier physical chemical studies on acid solutions of collagen led to much higher molecular weights, above 1,000,000 (M'Ewen and Pratt, 1953; Gallop, 1955a). There is reason to believe that differences in preparation were responsible for this discrepancy. Gallop (1955b) has shown that gentle warming of an acid solution of ichthyocol will reduce the molecular weight to 70,000 with an abrupt fall in optical rotation and an irreversible loss of ability to reconstitute fibrils.

The term 'tropocollagen' does not imply a precursor role. These particles may be obtained either by dissolving fibrils or by extracting them from the tissues before polymerization; in the latter case they would be precursors of the fibrils.

Because native collagen fibrils are relatively insoluble at neutral pH the discovery of a form of collagen extracted from fresh connective tissue by cold neutral salt solutions (Highberger *et al.*, 1951; Gross, *et al.*, 1955) suggested the presence in the tissue of a form of collagen in the dispersed state, perhaps dissolved in the ground substance. Studies by Harkness *et al.* (1954) on the turnover rate of isotopically labelled collagen in rabbit skin and by Jackson and Slack on carrageenin-induced granulomata in rats, indicated a relatively large and rapid turnover in the neutral salt-extracted collagen, much less in the acid-extracted fraction, and practically none in the insoluble residue. The metabolic activity of the neutral salt soluble fraction indicates the likelihood of its being a precursor of the fibrils. If this hypothesis is correct there should be considerable variation in the amounts of neutral salt-extracted collagen related to growth and certain disorders manifested in the connective tissue such as scurvy.

The aim of this paper is to describe some of the properties of cold neutral salt extracts of fresh connective tissue, and to illustrate the effect of growth rate on the extracted components.

GENERAL METHODS OF PREPARATION AND ANALYSIS

The studies reported here were all performed on albino guinea-pigs ranging in weight from 300 to 500 grammes. The animals were

fed with standard guinea-pig pellets plus a large daily supplement of fresh lettuce and 50 mg. of ascorbic acid *per os* twice weekly. Weight was recorded daily. Fresh dermis was coarsely ground, suspended in 2 volumes of cold extracting medium, shaken for 18 hours, centrifuged at 50,000 g. for 30 minutes, and the supernate filtered through coarse, medium and fine sintered glass with suction. All operations were performed at about 5° C. Prior to viscosity determination samples were centrifuged again at 50,000 g. for 3 hours. Aliquots of the filtrates were retained for analyses and for electrophoresis and ultracentrifuge studies.

Viscosity was determined at 5° C. in Ostwald capillary viscometers with flow times of 68 and 167 seconds at this temperature. Electrophoresis in a Perkin-Elmer apparatus was performed at 3° C. on aliquots dialysed against a veronal-citrate-NaCl buffer, $\Gamma/2 = 0.2$, pH 8.6.

Ultracentrifugation was performed in the Spinco analytical centrifuge at 56,100 g. at 5 to 8° C. Two samples were usually run simultaneously with the aid of a wedge cell.

Chemical analyses[1] were performed as follows:

Hydroxyproline	– Neuman and Logan (1950)
Tyrosine	– Bernhardt (1938)
Hexose	– Friedman, orcinol (1949)
Hexosamine	– Boas (1953) modification of Elson and Morgan (1933)
Uronic acid	– Fishman *et al.* (1951) (naphthoresorcinol)

The electrophoretic pattern of a cold NaCl or phosphate ($\Gamma/2 = 0.45$, pH 7.6) extract of guinea-pig corium qualitatively resembled that of guinea-pig serum except for the high, slow-moving and slowly diffusing peak in the γ-globulin fraction. There were obvious differences in relative amounts of the different proteins as is readily seen by comparing the ascending pattern of serum (Fig. 1*a*) with that of the extract (Fig. 1*b*). Extracts prepared with 0.14*M* NaCl revealed essentially the same pattern with a much smaller collagen peak (Fig. 1*c*). Warming extract (1*b*) at 37° C. for two hours produced a rigid opaque straw-coloured gel (Gross *et al.*, 1955).

[1] All analyses were performed on aliquots of extracts flash-heated to reduce the viscosity to a low level.

Uronic acid values are only good for comparative purposes. Pure hyaluronic and chondroitin sulphuric acids yielded only 20 per cent of theoretical yield of uronic acid with the colorimetric method used. The Dische reaction and decarboxylation methods were not applied because of the mixture of unknown substances present.

This was centrifuged at high speed and the supernatant fluid again examined by electrophoresis. Fig. 1*d* shows the major change to be the near-loss of the high spike in the γ-globulin fraction. This solution was no longer viscous and its hydroxyproline content had fallen from 131 γ/cc. to 33 γ/cc. The lost

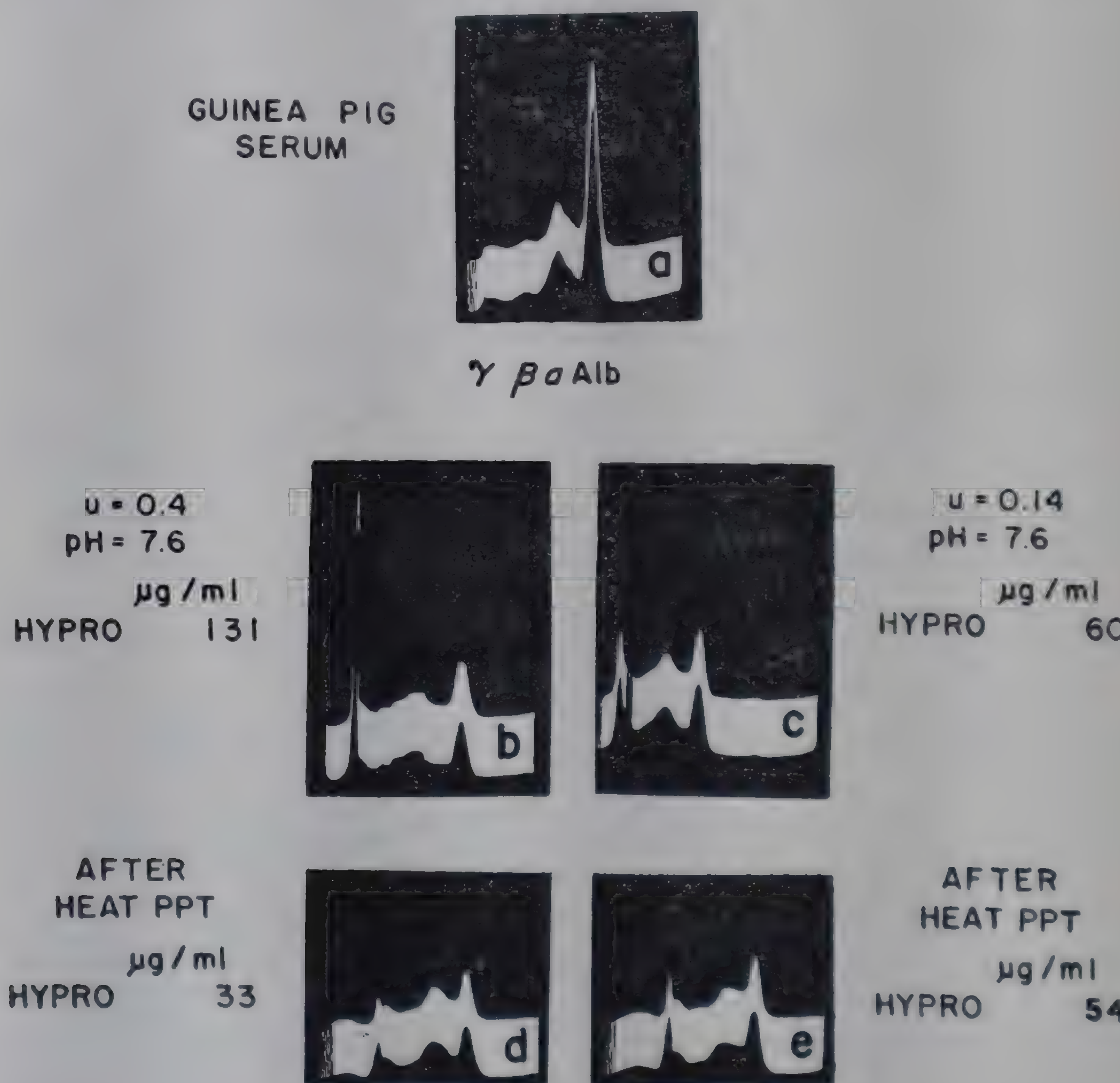


FIG. 1
Electrophoretic patterns (ascending limb) in Veronal citrate – NaCl, pH 8.6, $\Gamma/2 = 0.2$ of cold phosphate extracts of fresh guinea-pig dermis.
(*a*) Guinea-pig serum.
(*b*) Dermis extract, phosphate, $\Gamma/2 = 0.45$, pH 7.6.
(*c*) Dermis extract, phosphate, $\Gamma/2 = 0.14$, pH 7.6.
(*d*) Supernatant of extract (*b*) after heat gelation and removal of collagen precipitate.
(*e*) Supernatant of extract (*c*) after heat gelation and removal of collagen precipitate.

hydroxyproline was found in the fibrous precipitate. Electron microscopy of this material revealed it to be composed primarily of collagen type fibrils having the usual 640 Å period plus much

amorphous debris. It thus appeared that the slow-moving, high, hypersharp peak was the extracted collagen fraction. Fig. 1*e* is the pattern obtained from the supernatant after heating the low ionic strength extract (1*c*) for the same period. Only a small amount of collagen was precipitated, most likely because of the much lower concentration.

Ultracentrifuge patterns obtained before and after heat precipitation also yielded similar results. Here the extracted collagen is manifested as a hypersharp, slowly sedimenting boundary as shown in Fig. 3.

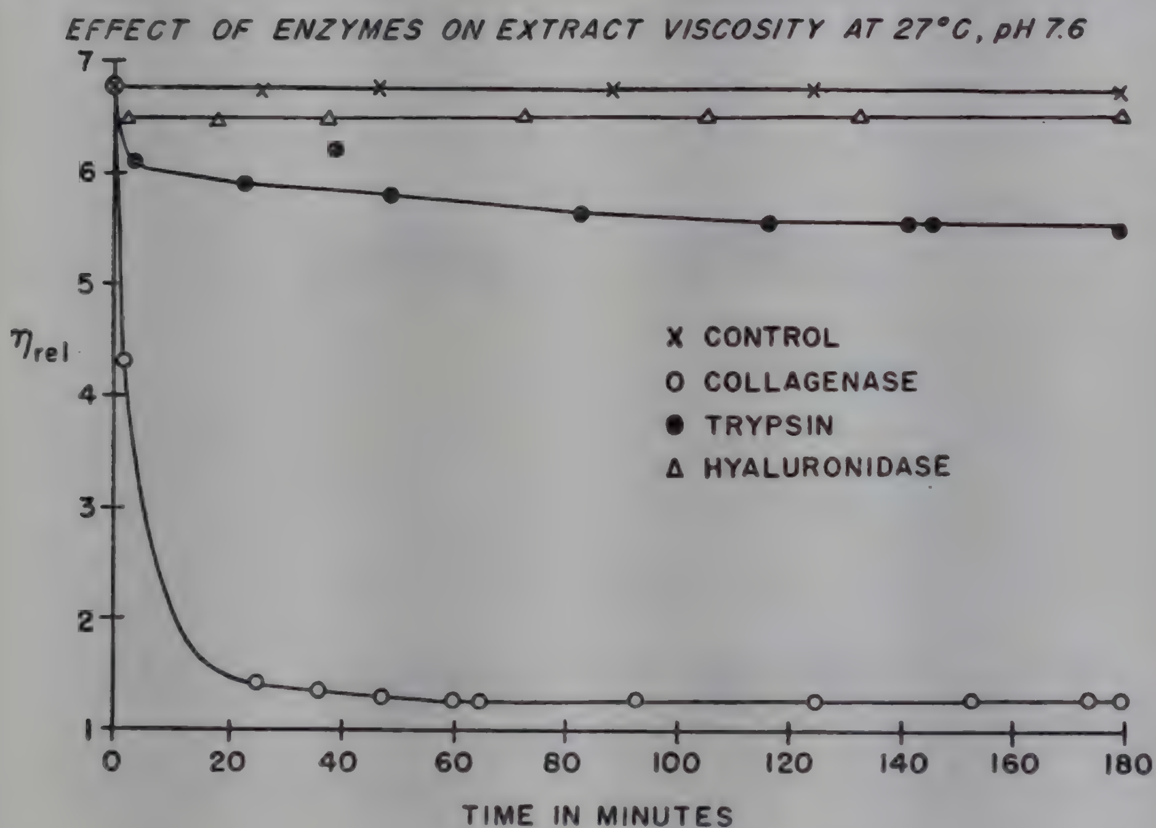


FIG. 2

Effect of enzymes on viscosity of 0.45*M* extract at 27° C. as a function of time. The extract was dialysed against phosphate $\Gamma/2 = 0.45$, pH = 7.6 prior to experiment.

In an effort to characterize these extracts further, the effect of collagenase, trypsin and hyaluronidase on viscosity, sedimentation and electrophoretic pattern was examined. A 0.45*M* NaCl extract was dialysed vs. phosphate buffer $\Gamma/2 = 0.45$, pH 7.6, and split into four equal aliquots. The temperature was raised to 27° C. in a water bath and collagenase (1 mg./cc.), trypsin (1 mg./cc.) and hyaluronidase (1 mg./cc.) were added to each of three tubes (dissolved in small volumes of buffer) and the same volume of buffer alone added

to the fourth tube. Viscosity readings were made at frequent intervals in an Ostwald viscometer at 27° C. Results are shown in Fig. 2. Viscosity fell rapidly in the collagenase-treated tube to a minimum within 20 minutes and levelled off at a value close to that of the buffer. There also was a rapid but much smaller fall in viscosity of the trypsin-treated sample to a minimum in 5 minutes followed by a slow, linear decrease. A small, but repeatable, and nearly instan-

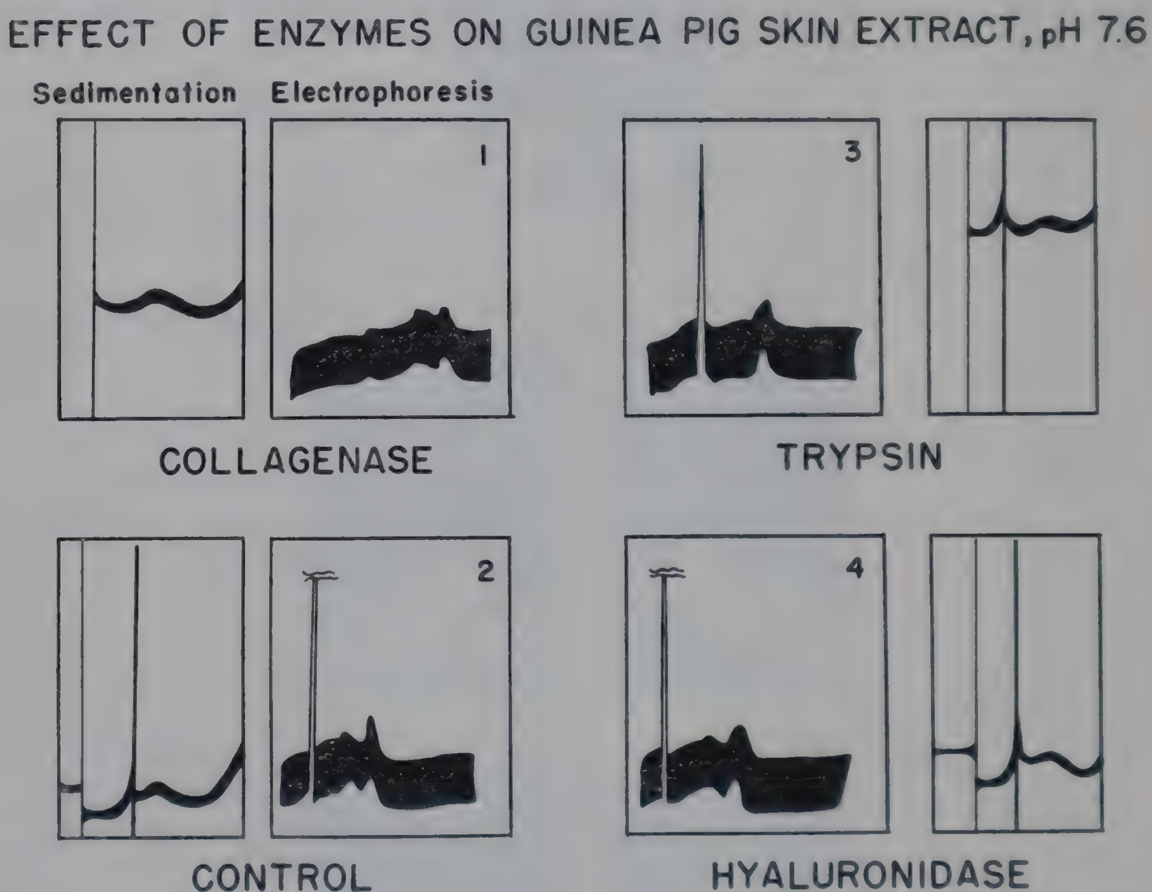


FIG. 3
Ascending electrophoretic patterns and ultracentrifuge tracings of enzyme-digested extracts and control.

taneous, fall was noted in the viscosity of the hyaluronidase-treated sample as compared with the control. Electrophoretic and sedimentation patterns of all four preparations after enzyme treatment are illustrated in Fig. 3. It is evident that the collagenase attacked the collagen peak primarily, although there appeared to be some modification of the rest of the pattern. Trypsin seems to have reduced the height and increased somewhat the diffusability of the collagen peak. There is also obvious although incomplete destruc-

tion of the non-collagenous components. Hyaluronidase seems to have no obvious effect on either pattern.

Attempts to heat-precipitate these preparations at 37° C. produced neither precipitate nor gel in the collagenase-treated sample, a heavy, fibrous, non-gelled precipitate in the trypsin-treated preparation, and the typical rigid, opaque gel in both hyaluronidase-treated and control samples. The most interesting result here is the effect of trypsin. It has long been stated that this enzyme does not attack collagen although Gustavson has demonstrated a lowering of the shrinkage temperature of fibrous collagen as a result of such treatment. The effect in these experiments was manifested by a rapid but limited fall in viscosity, small changes in the electrophoretic appearance of the collagen boundary and also by the absence of gelation on warming to 37° C. The fibrous precipitate which did form, contained both non-striated and striated fibrils; however, more careful electron microscope study is in order. This experiment was repeated on a neutral phosphate solution of calf-skin collagen prepared by dialysing an acetic acid extract of calf corium against phosphate buffer as described by Gross (1956). Extracts prepared in this manner contain relatively 'pure' collagen. A single hypersharp boundary was obtained in the ultracentrifuge and electrophoretic apparatus at both acid and alkaline pH. However, small amounts of non-collagenous material is associated as evidenced by the presence in the dialysed lyophilized material of 0.9 per cent tyrosine, 1 per cent hexose and 0.2 per cent hexosamine. The effect of the enzymes on viscosity and heat gelation were essentially the same as that on the crude tissue extract. The question as to whether or not trypsin attacks the collagen molecule directly in a specifically restricted manner or through an intimately associated non-collagenous substance, awaits repetition of this experiment on a more pure collagen preparation.

An important question raised is the source of the collagen extracted. Dilute NaCl does produce a certain amount of swelling of collagen fibres, more than that of pure water; thiocyanate, a strong hydrating agent, produces even more, while sulphate ions which have the least hydrating effect (reflected in its position in the lyotropic series) produce little swelling if any at all (Gustavson, 1956). Thus, if the neutral salt extracted collagen were derived from the formed collagen fibrils, the most effective extractant should be thiocyanate and the least should be sulphate. Three equal aliquots of one fresh

ground guinea-pig corium were extracted with cold NaCl, NaSCN and Na_2SO_4, all $\Gamma/2 = 0.45$. The most effective medium was Na_2SO_4, the other two were somewhat less effective, but about equal, as manifested by viscosity and hydroxyproline content of the extracts. This experiment strengthened the view expressed earlier that neutral salt-extracted collagen does not derive from the ordinary fibrils. It is worth noting here that saturated Na_2HPO_4 used by Harkness *et al.* (1954), is a much less effective extractant than any of the three salts mentioned above. Borate at ionic strength

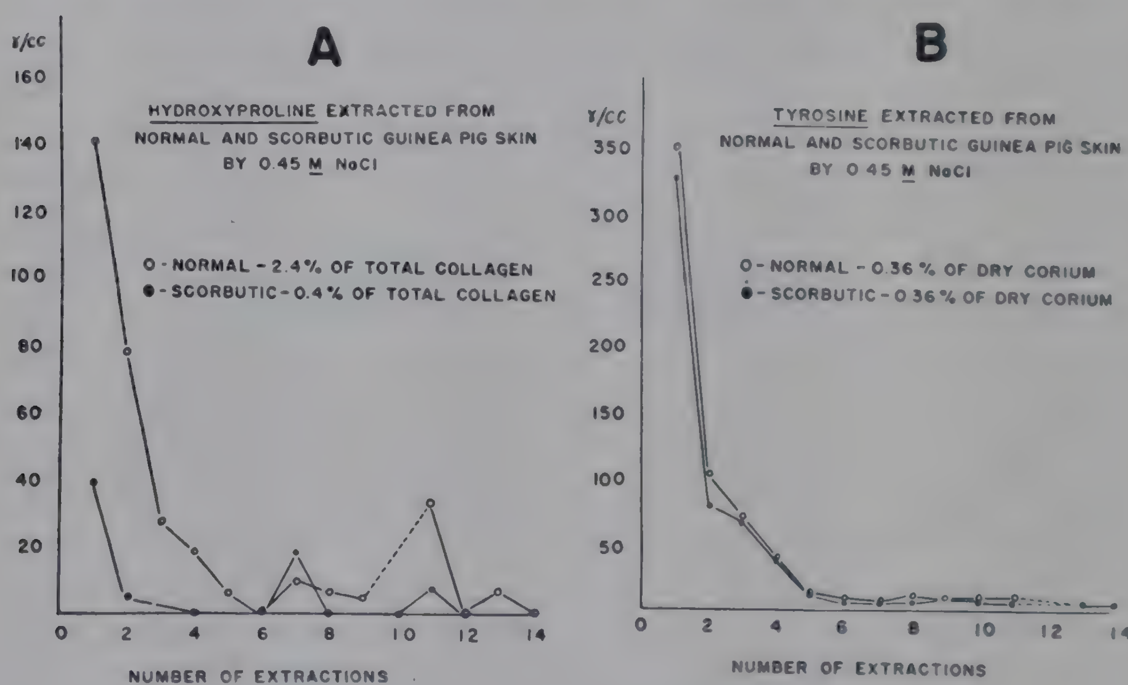


FIG. 4

Non-dialysable hydroxyproline (collagen), A, and tyrosine (non-collagenous protein), B, extracted from a scorbutic guinea-pig corium as compared to that from the skin of a pair-fed control. Extracting medium was $0.45M$ NaCl.

0.45, pH 7.6 extracts negligible amounts of collagen under these conditions.

It was reasoned that if the neutral salt-extracted collagen was truly a precursor of the fibrils, this fraction should be involved in the scorbutic process. If scurvy is characterized by an inability to synthesize collagen there should be either a deficiency or an alteration in properties of the precursor. A group of young guinea-pigs was placed on a vitamin C-free diet and another group of controls was pair-fed with them. A daily weight record of all animals was kept. After 23 days the scorbutic animals were nearly moribund. Histologic assay of the mandibles revealed total scurvy in the test animals, and

none in the controls. The skins of the scorbutic and control animals were processed as described, using cold 0.45*M* NaCl as an extractant. The tissues were extracted repeatedly until hydroxyproline and tyrosine were no longer detected in the solution. Fig. 4*a* illustrates the substantial difference in extractible hydroxyproline between a scorbutic animal and its pair-fed control. Fig. 4*b* reveals no difference

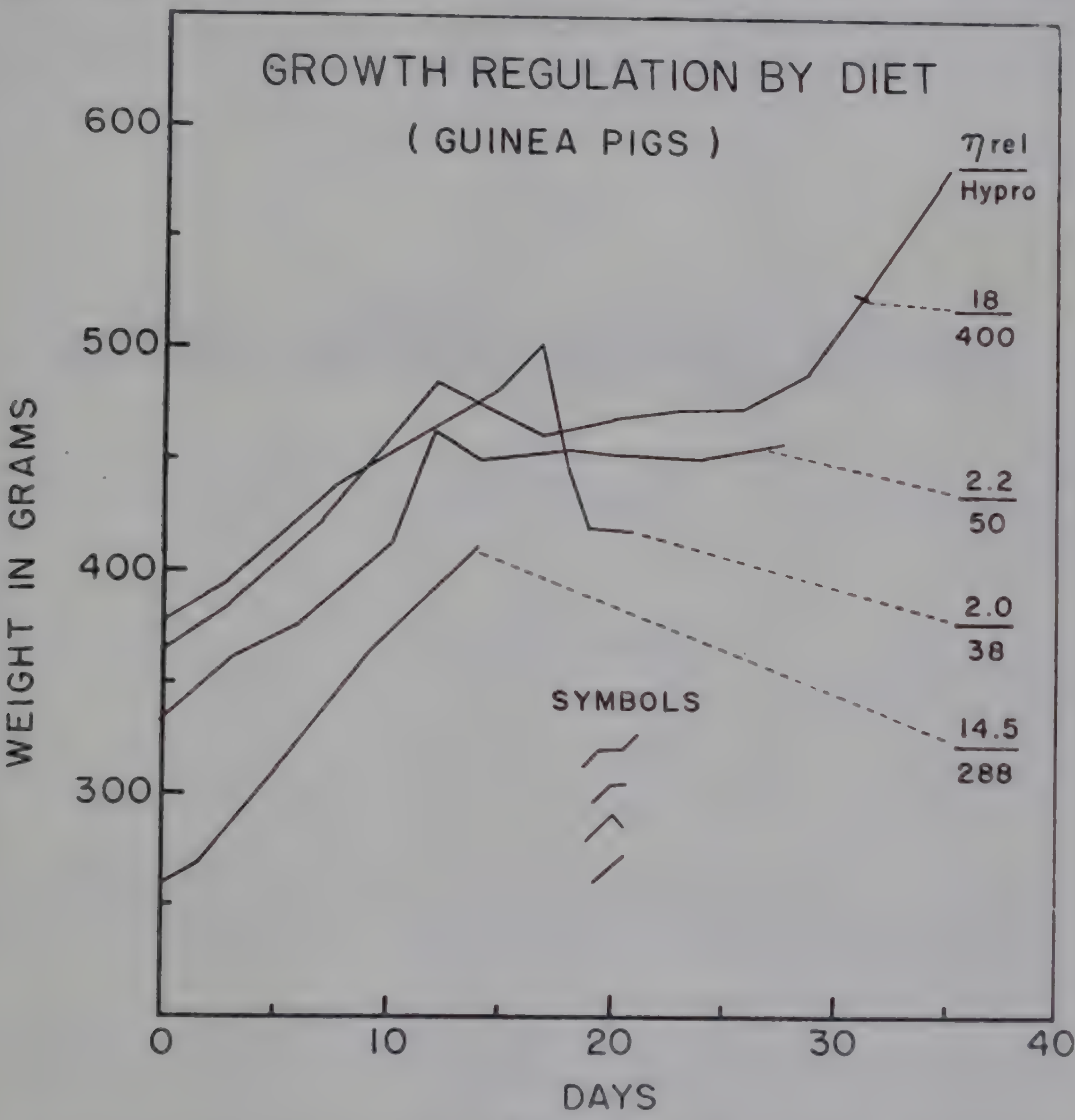
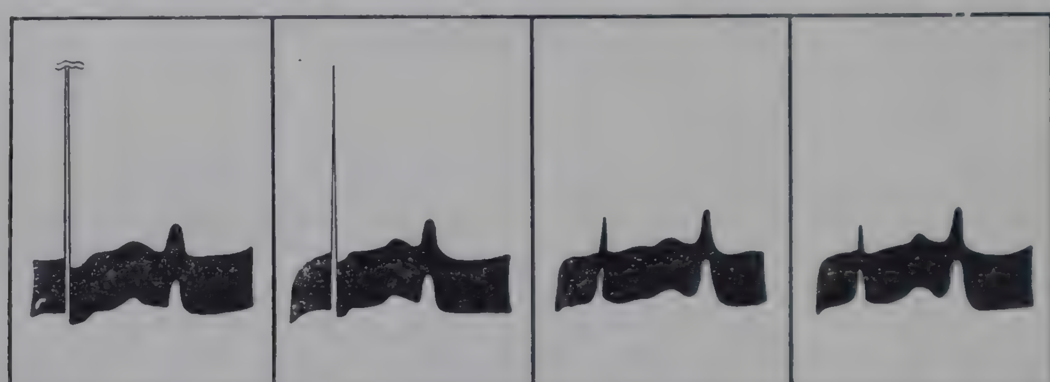


FIG. 5
Representative protocols of growth curves of guinea-pigs used in series of experiments on the effect of growth rate on neutral salt-extracted collagen.

in the amount of extracted tyrosine, a measure of non-collagenous protein. However, a similar study carried out on rat skin, kindly furnished by Dr. Henry Goldman from animals used for other purposes in a nearly identical dietary experiment, revealed a similar, although somewhat less marked difference in extractible collagen

between growing and non-growing rats. It was evident that growth rate was an important factor in the scurvy experiments and review of the protocols revealed that the pair-fed controls were still growing very slowly during their last five days, while the scorbutic animals were losing weight rapidly. However, physical chemical analyses showed changes which could be distinguished from those related to growth. These are now under study.

The following experiments were devised to evaluate the effect of



GROWTH CURVE		2	4	6
η_{rel}	17.1	3.6	2.3	2.0
HYPRO (μg/ml.)	388	89	54	49

Fig. 6

Electrophoretic patterns (ascending limb), viscosity and hypro content of extracts from the skins of guinea-pigs starved for varying periods of time after a long period of active growth. Symbols indicate characteristics of weight curve. Numbers on symbols indicate the number of days of weight loss.

growth on connective tissue components, particularly collagen extracted with cold neutral salt solutions.

Guinea-pigs of about 300 grammes in weight were allowed to grow rapidly for a period of 10 days on an *ad lib.* diet (Group A). They were sacrificed during the period of rapid growth. Another group (B) had growth interrupted by a sharp diminution of intake so that they remained practically static in weight for 8 days, then sacrificed. A third group (C) prepared in a similar manner to that of Group B was then allowed to grow rapidly again for 8 days after the period of static weight before sacrifice. A fourth group (D) was

starved for two days after the rapid growth period, with sharp loss in weight. A fifth (E) and sixth (F) group handled the same way, were starved for 4 and 6 days, respectively. All animals were well supplemented with ascorbic acid. The usual extracts were prepared, dialysed against cold saline, examined viscometrically and analysed for hydroxyproline, tyrosine, hexose and hexosamine and uronic acid. One representative extract from each group was studied in the ultracentrifuge and electrophoresis apparatus.

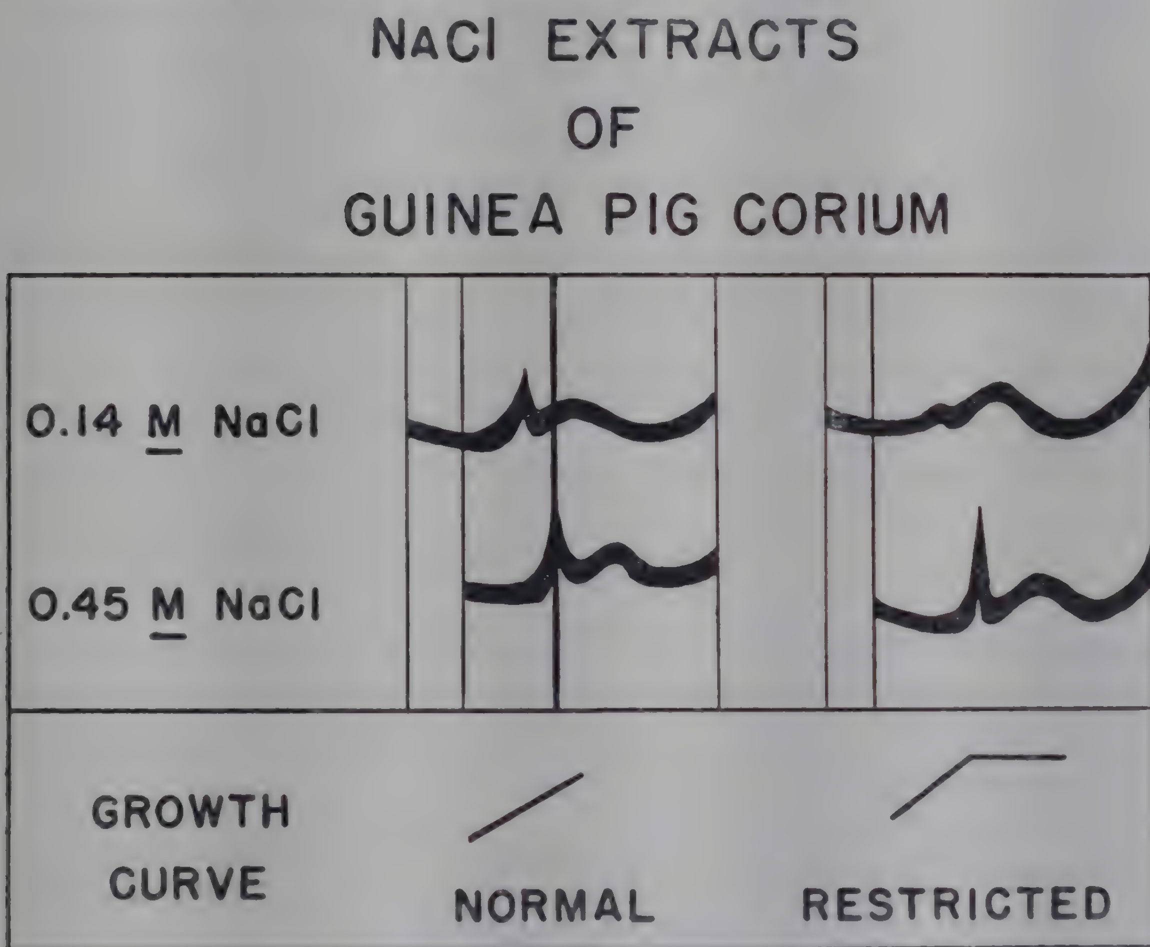


FIG. 7
Ultracentrifuge tracing of pattern obtained from extract of skin of rapidly growing guinea-pig as compared to that of a non-growing animal.

The results within each group were quite consistent. Fig. 5 illustrates one representative growth curve from each group and the viscosity and hydroxyproline contents of the extract from the skin of that particular animal. Fig. 6 allows comparison of electrophoretic patterns, viscosity and hydroxyproline data from extracts of representative animals of Groups A, D, E and F to show the effect of weight loss. Starvation for two days produced a marked drop in

viscosity and hydroxyproline. This is manifested in the electrophoretic pattern by a fall in the height of the collagen peak; the rest of the pattern is relatively unaltered. Four days of rapid weight loss results in further decrease in these same parameters with little further change in six days. Fig. 7 compares the sedimentation patterns of the extracts of a normally growing animal of Group A and a non-growing animal of Group B. Both low and high ionic strength

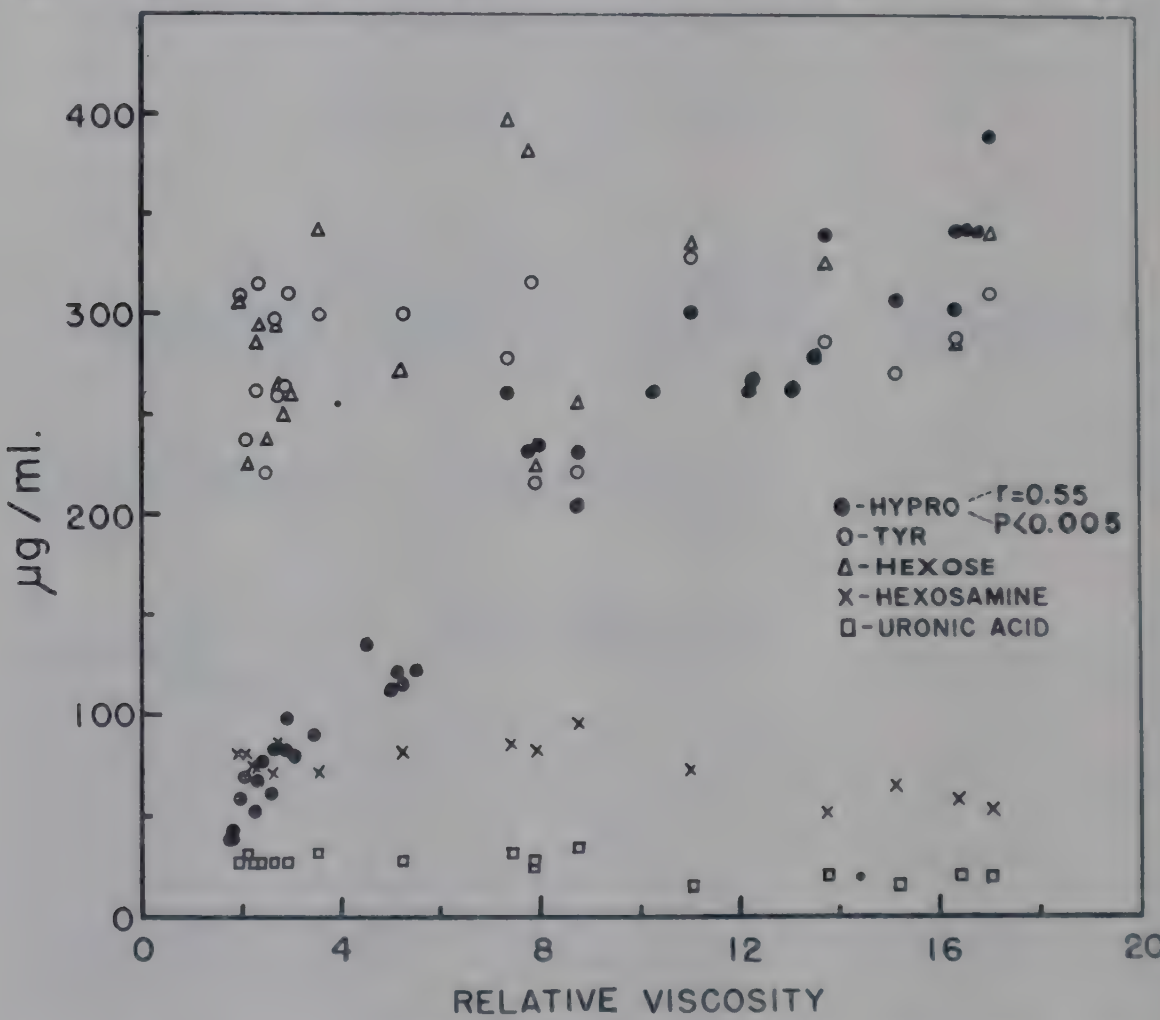


Fig. 8

Scatter diagram plotting amounts of extracted, non-dialysable hydroxyproline, tyrosine, hexose, hexosamine and uronic acid as a function of viscosity. It is evident by simple inspection that only the hydroxyproline correlates directly with viscosity.

extracts are compared. The sharp, slow-moving boundary representing the collagen fraction is markedly diminished in both types of extracts in the static weight animal. Viscosity and hydroxyproline content are commensurately low. Animals in Group C, having been allowed to resume growth for 8 days after an equal period of static weight yielded skin extracts of high viscosity and collagen content.

It is evident from these data that the amount of collagen extracted from the guinea-pig skin by cold neutral salt solutions is markedly dependent on growth rate. Two questions of considerable interest were the effect of growth on the carbohydrate and non-collagenous protein components of the extracts and the identity of the factors contributing to the viscosity of the extracts. Both questions are answered by plotting all the analytical data against viscosity for all the experiments, producing a scatter diagram (Fig. 8). Simple inspection reveals that the only factor showing a direct and nearly linear correlation with viscosity is hydroxyproline content. A similar relationship was found for glycine and proline, major constituents of collagen. Non-dialysable tyrosine, hexose, hexosamine and uronic acid are not significantly influenced. It should be emphasized, however, that these analyses only measure the quantity of substances present; they reveal nothing concerning properties such as configuration or degree of polymerization.

It would thus appear that the factor in cold neutral salt extracts most sensitive to changes in growth rate, is the dissolved collagen and that this component is responsible for extract viscosity.

DISCUSSION

The case for the precursor nature of neutral salt-extracted collagen is further strengthened by the data presented here, namely that the amount of extractible collagen correlates closely and directly with growth rate, that only two days of starvation with concomitant weight loss results in a many-fold diminution in the amount of salt-extracted collagen. 'Precursor' is here defined as the newly synthesized collagen molecules or small aggregates which have not yet been polymerized in the form of the native 640 Å banded fibrils but are destined to be so polymerized.

The evidence at hand from the isotope studies of Harkness *et al.* (1954) and Jackson (this symposium) on the turnover rate of alkaline and neutral salt-extracted collagen is strongly in favour of this possibility. That this fraction is readily polymerized *in vitro* into typical collagen fibrils indicates that it is a 'normal' collagen and probably not involved in a separate metabolic cycle for eliminating a faulty protein.

At this time there is no good reason to believe that this is the only precursor form. Certainly within the cells there must be peptide

chains in process of synthesis. It is hypothesized here that such incomplete molecules are incapable of being polymerized into fibrils and it is probably the fully formed and functional molecule which is 'secreted' into the extracellular ground substance where polymerization to fibrils occurs. An important question in this connection, as yet unanswered, is whether or not enzymatic modification of this particle is required before polymerization can occur, i.e. a process similar to that involved in the fibrinogen-fibrin transformation. Work is in progress on this phase of the problem.

Preliminary studies in this laboratory by viscosimetric methods indicate that the collagen particles in cold neutral salt extracts of fresh skin are highly asymmetric with axial ratios of the order of 300:1. It is, however, not inconceivable that partial polymerization or aggregation has already occurred in these extracts.

Whether or not any of the other carbohydrate or protein components of connective tissue are actually involved in the polymerization process in the formation of fibrils is, in the opinion of this writer, unanswered at this time. It is possible to form typical collagen fibrils *in vitro* in the absence of anything identifiable as an acid mucopolysaccharide such as hyaluronic acid or chondroitin sulphate. There seems to be a small quantity of sugar tightly associated with collagen, mostly hexoses in concentrations in the range of 0.5 per cent, and smaller amounts of hexosamine. No uronic acid has been detected in such preparations; inadequacy of the analytical and chromatographic methods employed cannot be excluded, of course. A minimal figure of 0.3 per cent tyrosine has been obtained in this laboratory for dissolved collagen capable of being reconstituted into fibrils. Whether or not these substances are integral parts of the molecule or just strongly adsorbed in a structurally irrelevant way so far as fibrilogenesis is concerned, is still a question.

A possible physiological function of the neutral salt-extracted collagen, aside from its probable role as a precursor, is suggested by the high viscosity it imparts to its solutions. If all the extracted collagen is present in the ground substance in a dispersed state rather than in some kind of fibrillar form, its concentration in the extracellular fluid in actively growing animals would be well above 1 per cent. Such a 'solution' of collagen, at least in the test tube, is nearly a gel; it has the consistency of thick molasses. The physical properties of the ground substance should then be strongly dependent upon the amount of neutral salt extractible collagen. In non-growing animals

then, there should be a marked difference in viscosity and other properties, of ground substance as compared to those of actively growing animals.

It is important, however, to emphasize that the neutral salt extraction does not remove all the extracellular substances, and the properties of such extracts may not necessarily reflect faithfully the properties of the intact tissue.

Conclusive correlation between the properties of tissue extracts and the physiological roles they may play awaits the devising of adequate methods for study of the behaviour of these components in the intact fresh tissue.

GROUP DISCUSSION

In reply to a question by Dr. Astbury, Dr. Gross said that the salt extract of scorbutic skin did not precipitate at 37° C. The collagen in the salt extract of normal skin probably differs from Doty's acid extracts of collagen in that there was some evidence for aggregation in the former. Recent physico-chemical studies he has made on collagen in neutral salt solution, even after prolonged high-speed centrifugation, indicate the presence of tropocollagen aggregates rather than separate units. Doty took particular care to remove aggregates from his material by prolonged ultracentrifugation, but in the case of the neutral salt solution such centrifugation was not effective.

Dr. D. S. Jackson said that he and Dr. Fessler had purified collagen from neutral extracts of rabbit skin by salt precipitation and had found that the purified material behaved in solution as rigid rods in varying degrees of aggregation. The length was 4000-6000 Å and the degree of asymmetry about 40, indicating a width of 100-200 Å. This had been confirmed by electron-microscope work. Solutions of this material precipitated on warming to 37° C. and also, more slowly, at lower temperatures. The best electron-microscopic pictures were obtained on material which had been allowed to precipitate very slowly in the cold room. This material was insoluble at neutral pH but soluble in dilute acetic acid. He also gave results of amino-acid analyses on neutral and acid extracts and also on the insoluble residue. He suggested that the neutral extract contains an extra material which would account for the lower glycine and hydroxyproline contents and the higher tyrosine, serine, threonine and histidine contents. This might be a contaminant or it might be important in the precipitation of this fraction.

Dr. Fitton Jackson suggested that high tyrosine content might be due to contamination by intracellular material.

Dr. D. S. Jackson pointed out that his neutral salt extract had been purified and that any such contaminant would have to be present in such large amounts that it would easily be detected by electrophoresis.

Dr. Fitton Jackson said that one would expect less contamination in old material which contained fewer cells.

Dr. Gross pointed out that collagen fibrils could be dissolved in dilute acetic acid and then dialysed against cold neutral salt solution; the solution thus obtained had similar properties to a neutral salt extract of collagen. He further stated that the presence of very small amounts of arginine or urea delays the heat precipitation of the neutral salt extract and that this effect can be reversed by iodide or thiocyanate.

Table I

SOLUBILITY OF COLLAGEN AND PROCOLLAGEN IN DIFFERENT SOLVENTS

Solvent	*Per cent of dissolved nitrogen* *Collagen* 1st Extr.	2nd Extr.	3rd Extr.	4th Extr.	Total	*Procollagen*
Phenol (80 per cent in water)	18.8	—			18.8	easily soluble
Lithium iodide (50 per cent in water)	7.5	3.0	1.6		12.1	easily soluble
Urea (saturated solution in water)	2.5	2.3	3.2		10.0	completely but not easily soluble
Thiourea (saturated solution in water)	1.1	2.5	5.5	4.2	13.3	completely but not easily soluble
Guanidine carbonate (saturated solution in water)			insoluble			insoluble
Guanidine iodide			insoluble			insoluble

Dr. D. S. Jackson said he had been unable to detect the splitting off of lower molecular weight peptides during precipitation of neutral salt extracts such as occurs in the fibrinogen-fibrin transformation and furthermore that the precipitate could be dissolved and reprecipitated and that this could be repeated several times.

Dr. Gustavson said that the setting of gelatin solution was markedly impaired by small amounts of arginine but not by the addition of hydroxyproline. Grabar and Morrel suggested that the arginine added may compete with the arginine residue (in collagen or gelatin) as to its function as a site for a strong cross-link in collagen. The very high pH of the guanidyl group in collagen may be due to its partial inactivation by such a link which will require great alkalinity for its rupture. He also compared the low hydroxyproline content of neutral salt-soluble collagen

with that of collagen in healing wounds in scorbutic animals and also in the skin collagen of certain teleostei (cod) and said that the lack of hydrogen bonds involving hydroxyproline might account for the solubility and low strength of these structures.

DR. GROSS referred to a theory propounded first by Robertson that in collagen of scorbutic animals the proline was not properly hydroxylated.

Following up a point raised by Dr. Baló, DR. GRASSMANN said that both acid soluble and neutral salt-soluble collagen were precursors of collagen, but in different senses. Tropocollagen has a very rapid turnover rate whereas procollagen is a collagen formed in fibrils but not yet stabilized by cross-linkages. This stabilization of procollagen to the mature collagen seems to occur very slowly. Procollagen and collagen which has been purified by treatment with calcium hydroxide and trypsin differ in that the former is completely soluble in reagents such as phenol and urea which break hydrogen bonds whilst collagen is only partly soluble (Table I). He suggested that collagen is stabilized by cross-linkages other than hydrogen bonds.

DR. GROSS said that collagen becomes more insoluble when it is stored, even in the cold, for a long time. This might be due to thermal agitation causing the collagen molecules to assume a more stable configuration within the fibril. He suggested that this might also account in part for the increased insolubility of collagen that occurs on ageing under physiological conditions.

DR. NEUBERGER compared this to the ageing of colloidal suspensions.

DR. OREKHOVITCH said that there was no new formation of procollagen in scorbutic animals whereas control animals did produce procollagen although the amount of this was less than in normally fed animals. He suggested that the abrupt fall in procollagen observed by Dr. Gross in scorbutic animals was due to the non-production of procollagen. He further suggested that due to the buffer properties of tissue fluids the first extract of the tissue with weak acid solution might not be actually an acid extraction but would be equivalent to extraction with neutral salt. The first acid extract gave, after heating, two distinct peaks in the ultracentrifuge.

DR. GROSS, however, said that when skin was extracted by the Orekhovitch procedure, the pH of the original buffer was identical with that of the final extract.

THE FORMATION AND BREAKDOWN OF CONNECTIVE TISSUE

D. S. Jackson

Connective tissue growth has been studied intensively, histologically (see Cameron, 1952, for review of literature) and to a lesser degree electron-optically, but very few biochemical studies have been carried out. The recent report by Robertson and Schwartz (1953) that relatively large amounts of connective tissue are formed locally following the subcutaneous injection of carrageenin, seemed to offer a good opportunity of carrying out a biochemical study of the formation of both collagen and sulphated polysaccharides in developing connective tissue. A concurrent histological study was also carried out (Williams, 1956).

The methods used in this study have been described in detail elsewhere (Jackson, 1956a, b, c; Slack, 1956a, b).

QUANTITATIVE STUDIES

The connective tissue was fractionated into three collagen fractions, viz.: neutral salt-soluble collagen, citrate-soluble collagen and insoluble collagen. The sulphated polysaccharides were also fractionated, viz.:

(1) neutral salt extracted, (2) released following papain digestion, (3) present in the residue after papain digestion.

Collagen

All three collagen fractions were found, viz.: neutral salt soluble, citrate soluble and insoluble, and in addition a previously undescribed hydroxyproline-containing fraction was found in the water-soluble proteins. The total collagen concentration (Table I) increased slowly at first and more rapidly between the sixth and ninth days. Maximum concentration was reached at 14 days, after which the concentration fell off. However, since the wet weight decreased from days 7-9 onwards the absolute maximum amount of collagen was present about the ninth day.

At 3 days neutral salt-soluble collagen appeared (Table I). The concentration of this fraction increased up to the fifth day and remained constant for 2–4 days before rising again coincident with the decrease in the wet weight of tissue. At 3 days the major proportion of the neutral salt-soluble collagen was not precipitated by salt or by dialysis. This water-soluble hydroxyproline-containing material

TABLE I

VARIATION WITH TIME, AFTER INJECTION OF CARRAGEENIN, OF THE CONCENTRATION OF THE COLLAGEN FRACTIONS EXPRESSED IN MG./100 GM. WET WEIGHT OF TISSUE

Time	Wet weight tissue	Total collagen	Neutral soluble		Acid soluble	Insoluble
			Total	Water soluble		
Days	gm.	mg./100 gm. wet weight tissue				
3	19.4	14.9	5.5	4.7	0.2	9.2
5	37.9	74.3	18.8	9.7	11.2	44.3
6	56.7	189.3	15.5	6.0	18.2	155.6
7	70.2	333.2	18.9	9.2	28.9	285.5
9	25.5	857.7	58.9	20.3	86.9	711.9
14	23.5	986.1	103.3	41.2	115.1	767.7
21	8.7	1068.3	159.0	58.5	155.2	754.1
28	4.7	717.0	136.5	55.0	148.6	431.9

Total from 3 guinea-pigs

was present throughout the period of the experiment, the concentration/time curve being parallel to that for the total neutral salt-soluble collagen (Table I). The concentration of citrate-soluble collagen, although low at day 3, increased throughout the time of the experiment. The concentration of insoluble collagen also increased steadily up to 14 days, the most rapid increase being between the fifth and ninth day. After 14 days its concentration decreased steadily.

In Table II the results are expressed as per cent of total collagen, showing the relative proportions of the three collagen fractions. As insoluble collagen accumulates the relative proportions of the soluble collagens decrease until the weight of tissue begins to decrease when an increasing proportion of the total collagen is found to be in a soluble form.

TABLE II

RELATIVE PROPORTIONS OF COLLAGEN FRACTIONS EXPRESSED AS PER CENT TOTAL COLLAGEN

Time	Neutral soluble		Acid soluble	Insoluble
	Total	Water soluble		
Days	per cent total collagen			
3	38.4	30.7	1.6	60.0
5	25.3	13.0	15.0	59.7
6	8.4	3.3	9.6	82.0
7	4.5	2.2	6.9	88.6
9	6.8	2.3	10.1	83.1
14	10.5	4.2	11.7	77.8
21	14.9	5.4	14.5	70.6
28	19.0	7.7	20.7	60.3

TABLE III

AMOUNTS OF $SO_4^{=}$/100 GM. WET WEIGHT TISSUE IN THREE FRACTIONS OF GRANULOMA TISSUE AND TOTAL WET WEIGHT TISSUE OBTAINED FOLLOWING SUBCUTANEOUS INJECTION OF 50 MG. CARRAGEENIN

(Figures are means of at least three determinations on pooled tissue from three guinea-pigs at each time interval)

Time after injection of 50 mg. carrageenin	Wet weight tissue extracted	Salt fraction 1 0.2 M NaCl pH 7.4	Papain fraction 2 10 mg. papain/gm. tissue	Residue sulphate 3 by hydrolysis remainder	Totals 1, 2 and 3
Days	gm.	mg./100 gm. wet weight tissue			
3	13	23	11	5	39
5	18.5	15	12	5	32
5.5	33	15	13	6	34
6	38	12	13	6	31
7	33	9	11	5	25
9	8.5	7	6	7	20
14	8.5	4	5	8	17
21	3.5	2	3	7	12
28	2.0	1	4	8	13
Normal guinea-pig skin	—	7	9	3	19

Sulphated Polysaccharides

The total bound sulphate of the developing tissue is already at a maximum 3 days after injection of carrageenin (Table III). Salt extractable polysaccharide accounts for 60 per cent of the total at this time, its concentration falling rapidly thereafter. Sulphated polysaccharide released by papain digestion reaches its maximum concentration about the seventh day and falls steadily thereafter. The residue sulphate varies little throughout and would appear not to be concerned in the developing tissue.

ISOTOPE STUDIES

Collagen

The highest specific activity was found in the neutral salt-soluble collagen. Two hours after injection its activity was already high and reached a maximum between 8 and 12 hours (Fig. 1). The activity had fallen rapidly by 24 hours and more slowly thereafter, substantial activity still remaining after 16 days.

TABLE IV

Specific activities of $^{35}SO_4$ in counts/min./0.1 mg. $SO_4^=$ found in three fractions from carrageenin induced granuloma in guinea pigs; $Na_2\,^{35}SO_4$, 1 μc/gm. body weight, injected intraperitoneally 5 days after 50 mg. carrageenin subcutaneously. Radioactivities counted with 29 ±0.3 mg. carrier $BaSO_4$ as infinitely thick discs of 1 sq.cm. surface area.

Time after $Na_2\,^{35}SO_4$	*Salt Fraction 1 0.2 M NaCl, pH 7.4*	*Papain Fraction 2 10 mg. papain/1 gm. tissue*	*Residue Sulphate Fraction 3 by hydrolysis of remainder*
2 hours	12	14	26
6 ,,	354	23	22
8 ,,	462	44	20
12 ,,	672	128	22
24 ,,	586	327	19
2 days	408	363	13
4 ,,	53	245	8
9 ,,	22	199	12
12 ,,	28	143	7
16 ,,	10	115	6

The activity of citrate-soluble collagen was low at 2 hours and rose rapidly to a maximum at 12 hours, considerably below the maximum activity of neutral salt-soluble collagen. The activity/

time curve for insoluble collagen was a little lower than that for citrate-soluble collagen and reached a maximum after 24 hours. Activity had fallen after 48 hours, and after levelling out continued to fall from the fourth day on.

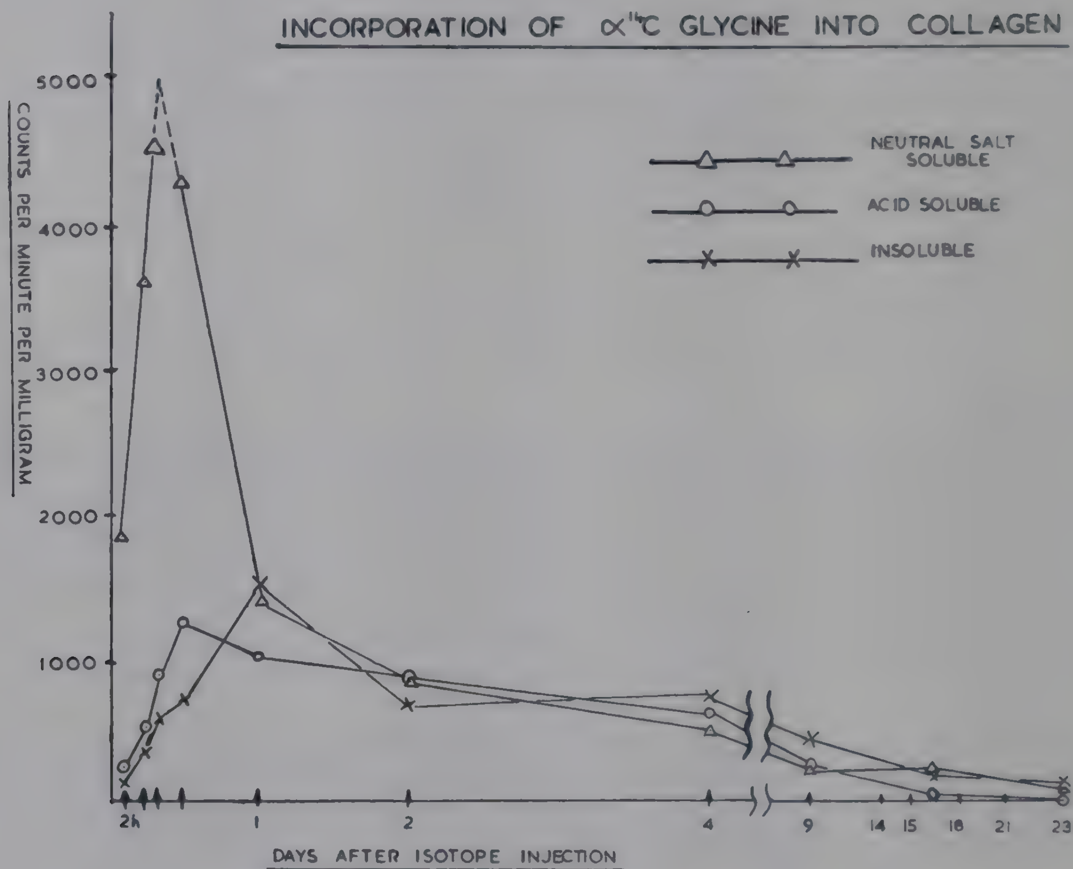


FIG. 1
Specific activity-time curves of collagen fractions:
△ Neutral salt-soluble collagen
○ Citrate soluble collagen
× Insoluble collagen

There was some variation in the activities of comparable fractions at comparable times, in experiments carried out at different times, but the activities of the different fractions relative to each other remained the same. Similar findings have been reported in the collagen fractions from rabbits injected intraperitoneally with [α^{14}C]-glycine (Harkness *et al.*, 1954).

Sulphated Polysaccharides

The specific activities of $^{35}SO_4$ in the three polysaccharide fractions are shown in Table IV. It will be seen that the most rapid incorporation of $^{35}SO_4$ is in the salt extracted fraction which reaches a maximum

activity in about 10 hours. The turnover rate of this fraction is also rapid being of the order of 36 hours. The maximum activity of the papain fraction is not reached until about 36 hours, the turnover rate being about 8 days, similar to that reported for the chondroitin sulphate of rat skin (Boström and Gardell, 1953) and of rabbit skin (Schiller *et al.*, 1955, 1956).

DISCUSSION

A comparison of the concentration/time relationship of total collagen (Table I) and total bound sulphate (Table III) suggests that in the early stages of connective tissue development only small amounts of collagen (probably of the soluble type) are present and relatively large amounts of sulphated polysaccharide. As the development continues the appearance of increasing amounts of insoluble collagen coincides with a decrease in the concentration of sulphated polysaccharides. This agrees with the histological findings of Stearns (1940a, b) and also the findings of Dunphy and Udupa (1955) in a combined histological and biochemical study of wound healing. During the pre-fibrous stage of development the main connective tissue components present are neutral salt-soluble collagen and the sulphated polysaccharides. The salt-extractable polysaccharide may be the precursor of that released by papain digestion, which may be closely associated with the fibrous collagens (Slack, 1956).

The finding that a proportion of the neutral salt-soluble collagen was water soluble was unexpected since negligible amounts of hydroxyproline-containing material were found in the same fraction from the skins of young rabbits (Harkness *et al.*, 1954; Jackson, unpublished data). The significance of this finding is not clear but it is possible that either this material represents an earlier stage of collagen synthesis or that it is a lower molecular-weight fraction of neutral salt-soluble collagen which is known to aggregate in solution to varying degrees (Jackson and Fessler, 1955).

Breakdown of Collagen

The concentration of insoluble collagen increases with time up to 14 days. Since the wet weight of tissue decreases after the seventh to ninth day, i.e. the tissue is breaking down and is being reabsorbed, synthesis and breakdown must be taking place simultaneously at least from the seventh day. It is possible that both take place simultaneously from the beginning but that synthesis predominates at

first and breakdown later. It has been found that carrageenin injected intradermally causes a rapid breakdown of the dermal collagen fibres without preceding granuloma formation (Williams, 1956). The increase in concentration of both the soluble fractions which occurs concurrently with the decrease in wet weight suggests that the breakdown of insoluble collagen occurs first by a disaggregation into the units which make up mature collagen fibres producing citrate- and neutral salt-soluble collagen, rather than by destruction of the constituent polypeptide chains although this will probably follow disaggregation. However, in normal connective tissue development, the mature collagen fibres are stable and metabolically relatively inert (Neuberger, Perrone and Slack, 1951). Hence the insoluble collagen produced by the method described here may be inherently unstable. It seems, however, in view of the results reported by Williams (1956), more likely that carrageenin first stimulates the formation of normal insoluble collagen and then stimulates some mechanism which leads to its removal.

Experiments with [$\alpha^{14}C$]-*Glycine*

The results of the [α^{14}C]-glycine experiments are in complete agreement with the suggestion put forward by Harkness *et al.* (1954) that neutral salt-soluble collagen and not citrate-soluble collagen as suggested by Orekhovitch (1952) is the true precursor of insoluble collagen. The more recent work of Orekhovitch (1955) reiterating his belief that citrate soluble collagen is the true precursor is invalidated because the method of extraction used would produce a mixture of the two soluble collagens; likewise the activity/time curve obtained would be the resultant of the curves for the two separate fractions.

Harkness *et al.* (1954) also suggested that citrate soluble collagen and insoluble collagen are not distinct groups of proteins but are both obtained from fibres deposited outside the cell. They also express the opinion that it is unnecessary for all the insoluble collagen to pass through the citrate soluble stage. The activity/time curves obtained in the present study support these ideas, since those of citrate soluble collagen and insoluble collagen are very similar and suggest that [α^{14}C]-glycine is incorporated simultaneously into both fractions.

There are some differences in detail between the present study and that of Harkness *et al.* (1954). The rate of incorporation of [α^{14}C]-

glycine into the neutral salt-soluble collagen is more rapid, maximum incorporation occurring at about 10 hours as compared with 24 hours for the same fraction from rabbit skin. The activity/time curves for citrate-soluble collagen and insoluble collagen are also different in the two studies, maximum incorporation into acid-soluble collagen occurring in about 12 hours as compared to 3 days for rabbit skin, and into insoluble collagen in 24 hours as compared to 8 hours. These differences may be due to species difference or more probably to the higher rate of growth of the carrageenin-stimulated connective tissue.

Harkness *et al.* (1954) believed that the comparatively high activities which they obtained for insoluble collagen at 8 and 24 hours were due to contamination with non-collagen protein of high activity. In this study this difficulty did not arise, the activity of insoluble collagen being low at 2 hours, when the activity of the neutral salt-soluble collagen was already very high (Fig. 1).

The persistence of radioactivity in the soluble collagens for as long as 16 days (Fig. 1) is probably due to the breakdown of insoluble collagen. As was shown above (Table I), the decrease in wet weight of the tissue from the seventh day onward is accompanied by a considerable increase in the concentration of soluble collagens. This fact, together with the similarity of the activity/time curves of all three fractions from the seventh to twenty-eighth day suggests that all these components are derived from insoluble collagen during its breakdown. Thus the formation of soluble collagens may be the first stage in the dissolution of collagen fibres.

The Morphological Origin of the Collagen Types

Before discussing the morphological origin, it will be well to review the question of nomenclature and define the terms used to describe the various collagen types. It has become customary to call the fraction extractable with acid buffers 'procollagen', the assumption being that it is the precursor of mature collagen fibres (Orekhovitch, 1952, 1955). This assumption is not now valid since it is more probable that collagen extracted with neutral salt solutions is the true precursor (Harkness *et al.*, 1954; Jackson, 1956a, b, c). Thus this fraction will be referred to as 'citrate soluble collagen'.

Greater amounts of collagen can be extracted from skin and tendon with dilute acids than with acid buffers (Bowes *et al.*, 1953, 1956; Jackson, unpublished data). Furthermore, treatment of tendon with

hyaluronidase greatly increases the proportion of this tissue extracted with dilute acetic acid (Jackson, 1953) and only slightly increases the proportion extracted with acid buffers (Jackson, unpublished data).

A further distinction must therefore be made between citrate-soluble collagen and collagen extracted with dilute acid, which will be referred to as 'acid-soluble collagen'. The residue remaining after extraction with neutral and acid buffers and which is usually extracted as gelatin will be called 'insoluble collagen'.

Citrate-soluble Collagen

The morphological origin of insoluble collagen is well known, but the origin of the two soluble types is less clear. Harkness *et al.* (1954) have suggested that citrate-soluble collagen is derived from collagen recently laid down, viz. from the outer layers of large fibres and from the thin argyrophilic fibres which are prevalent in developing connective tissue (Gross, 1950). It has also been suggested by Banga *et al.* (1956) that citrate-soluble collagen is an important constituent of large collagen fibres, playing an important part in the physical properties of the whole collagen fibre. Tustanowski *et al.* (1954, quoted by Banga *et al.*, 1956) believe that citrate-soluble collagen determines the physical properties of the mature fibre which, they suggest consists of a core of 'collastromin' which is structureless, on which are oriented fibrils of citrate-soluble collagen.

Preliminary experiments (Jackson and Williams, unpublished data) involving the histological examination of carrageenin-stimulated connective tissue following extraction with neutral and acid buffers (Jackson, 1956c) suggests that at least part of the citrate-soluble collagen is derived from the thin argyrophil fibres present in the early stages of connective tissue growth and often called reticulin. Following extraction with $0.2M$ NaCl, the thin argyrophil fibres no longer stain with silver, but stain red with the van Gieson stain like the thicker mature fibres. Extraction with citrate removes the thin fibres almost completely while leaving the thicker fibres apparently little affected. These findings also indicate that the reticulin of developing tissue is different from that found in the reticular tissues such as kidney basement membrane, since this is insoluble in acid buffers, and contains both lipid and carbohydrate (Kramer and Little, 1953; Windrum *et al.*, 1955).

Neutral Salt-soluble Collagen

The absence of silver staining after extraction with neutral salt suggests that the ability of the thin fibrils to take up silver is due to the presence of neutral salt soluble collagen on the surface of the fibril, which is not yet incorporated into the fibre structure. It has been shown by Schwarz (1956) by electron-optical studies of developing tissue that the silver particles taken up by this type of reticulin are on the surface of the fibre and are topographically related to the D bands of the fibres.

Since microscopically visible fibres are absent when neutral salt-soluble collagen is present (see Table I), this fraction is probably also present in the non-fibrillar part of the connective tissue.

It cannot be decided from the present study whether any of the neutral salt-soluble collagen isolated was originally intracellular. Studies by Porter and Vanamee (1949) using the combined techniques of tissue culture and electron microscopy, have shown that early fibrils are formed at the cell surface, and may be clearly connected with intracellular fibroglial fibres. These early fibrils reach a mature form extracellularly by accretion of material from the ground substance. These findings have been confirmed (Fitton Jackson, 1954a, b) in tissue culture of embryonic bone. It was not possible to decide whether the fibroglial fibres were actually intracellular (Fitton Jackson and Smith, 1955). However, collagen fibres fail to develop in tissue culture in the presence of anti-collagen serum (Robbins, Watson, Pappas and Porter, 1955), forming instead amorphous masses, having the tinctorial properties of collagen, which are probably antigen-antibody complexes. This would suggest that a non-fibrous precursor is secreted by the cell, forming fibrous collagen extracellularly. Cytoplasmic granules in connective tissue cells have been put forward as possible precursors of both amorphous matrix and collagen fibres (Fitton Jackson and Smith, 1955). Stearns (1940a, b) concluded on the basis of her observations of fibre formation in a transparent chamber in the rabbit ear, that these granules secreted a soluble precursor into the extracellular space, where this material was transformed into a fibrous form. It has recently been shown that neutral salt-soluble collagen can precipitate spontaneously into typical collagen fibres (Jackson and Fessler, 1955). It would seem probable that intracellular collagen is non-fibrous and is secreted into the extracellular spaces from which it can be extracted as a neutral salt-soluble collagen.

Insoluble Collagen

The end product of normal connective tissue formation is the thick collagen fibre which is largely insoluble in most salt solutions. There are probably several factors which determine the solubility properties of this type of collagen fibre. Gustavson (1955) has shown that stabilizing cross-linkages exist between the hydroxyl groups of hydroxyproline and the keto-imide groups of adjacent polypeptide chains. However, there are probably other factors involved. Jackson (1953) suggested that chondroitin sulphate might be a stabilizing factor, but later (Jackson, 1954) suggested that a mucoprotein other than chondroitin sulphate might play an even more important stabilizing role. This idea has been supported by evidence that a mucolytic enzyme which splits a mucoprotein present in tendon also reduces the stability of the tendon as measured by shrinkage temperature (Banga *et al.*, 1955). The same authors (Banga *et al.*, 1956) also suggest that citrate soluble collagen is a stabilizing factor since extraction with acid-citrate buffer reduces the shrinkage temperature by 12° and prevents the complete shrinkage-relaxation cycle characteristic of 'young' fibres, which is absent from 'old' fibres. It is possible, however, that prolonged soaking in acid buffer would itself affect cross-linkages and reduce the shrinkage temperature. It is noteworthy that 'young' human tendon swells considerably at acid pH without any collagen being dissolved (Banfield, 1952).

The Formation and Breakdown of Collagen

On the basis of the evidence available a tentative scheme for the formation and removal of collagen is put forward (Fig. 2). The role of the ground substance in the process is obscure. The fact that comparatively large amounts of sulphated polysaccharides are present before any visible fibres are formed may indicate that they provide the necessary environment for fibrogenesis.

The fibroblast apparently synthesizes a precursor, possibly the collagen molecule which has been called tropocollagen (Gross *et al.*, 1955). This is secreted by the fibroblast into the extracellular space from which it is extracted as neutral salt-soluble collagen. This can be handled in two ways.

(1) Form submicroscopic fibrils which can accumulate further particles of neutral salt-soluble collagen to form visible histological reticulin, i.e. citrate-soluble collagen, in which the cross-linkages are only fairly strong. They may also contain a citrate-soluble form of

non-collagenous protein only weakly linked to the collagen (Bowes *et al.*, 1956). Further particles of neutral salt-soluble collagen are added on, making the fibre thicker and longer. The cross-linking of the core of these fibres may now be very strong and may include mucoprotein, now only extractable with alkali (Bowes *et al.*, 1956). The outer fibres most recently laid down may still have only weak cross-links and still be soluble in acid buffers. Finally the whole fibre

A SCHEME OF FIBROGENESIS

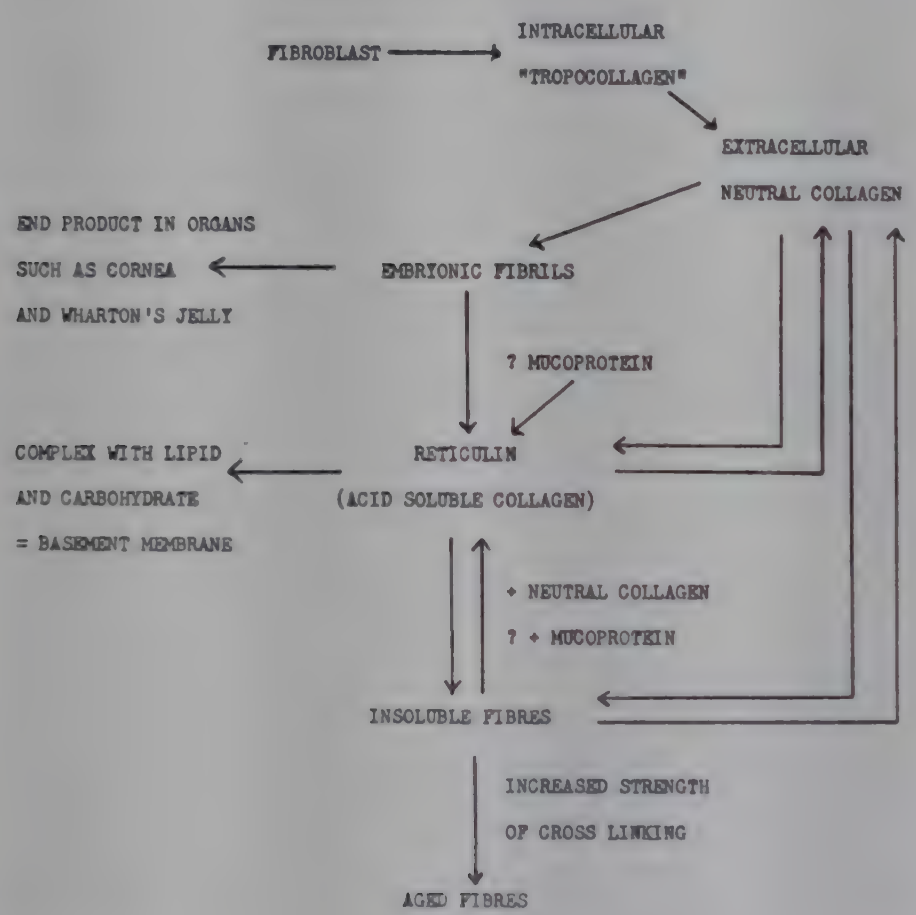


FIG. 2

matures with age, the cross-linkages increasing in strength and hence the proportion soluble in acid buffer decreasing.

(2) Once the thicker fibres form, the neutral salt-soluble collagen can add on directly increasing their size and thickness, so that at this stage citrate-soluble collagen need not be an intermediate between neutral salt-soluble collagen and insoluble collagen.

The first process would probably predominate in the early stages of connective tissue development and the second when the tissue is maturing.

In certain organs the process will stop at earlier stages. In such tissues as the nucleus pulposus, cornea and Wharton's jelly, the process goes no further than the sub-microscopic fibrils. Similarly in the reticular tissues the argyrophilic fibres do not develop further, possibly due to the formation of a complex with lipid and carbohydrate.

It would also appear from the work described above (see p. 67) that in some circumstances the process is reversible and the insoluble collagen finally formed breaks down, first forming the soluble types of collagen. Hence part of the process is shown as reversible in Fig. 2.

It is hoped that the experimental model described here will prove suitable for investigations into the factors which influence the formation of connective tissue and to discover at which of the stages of fibrogenesis these factors are effective.

ACKNOWLEDGMENTS

I am grateful to Professor J. H. Kellgren for his support and to Dr. H. G. B. Slack who carried out the work on the sulphated polysaccharides and kindly allowed me to use his data before publication and to Dr. G. Williams who did the histological study.

GROUP DISCUSSION

In reply to a question from Dr. Robb-Smith, Dr. Jackson suggested that neutral salt extraction removed the outer part of the argyrophilic fibres without dissolving the fibres completely and that it is the outer components of the fibres that are responsible for argyrophilia. He suggested that the silver is adsorbed on the surface of the fibre.

Dr. Gross said that treatment with trypsin also prevents the argyrophilia and suggested that the argyrophilia was a property of an extra-collagenous material.

Dr. Gross added that the injection of saline which spreads the collagen fibres apart, as observed by Ranvier and later by Nageotte, reveals the presence of branching argyrophilic fibres which cannot be seen in the compact tissue. He suggested that the immature 'reticulin' fibrils are never enlarged to collagen fibrils but are overwhelmed statistically by the vastly greater numbers of large collagen fibrils laid down as the animal ages. He suggested that argyrophilia might be due to mucopolysaccharides or lipids or similar non-collagenous components.

Dr. Jackson said that reprecipitated fibres of citrate soluble collagen which did not contain polysaccharide nevertheless may show argyro-

philia. He thought the argyrophilia was due to the outer part of the collagen fibre. Dr. Robb-Smith agreed with Dr. Jackson that it is important to distinguish between two types of 'reticulin', that of the basement membrane and the argyrophilic fibres in developing or regenerating connective tissue.

In reply to Dr. Meyer, Dr. Jackson said that as far as extraction procedure and analysis for hydroxyproline went, the neutral salt soluble collagen from granuloma is indistinguishable from that extracted from normal skin; but that he regards the material extracted from granuloma during the resorption process as a stage of breakdown of collagen and that from normal skin and from the early granuloma as a stage in the synthesis of collagen.

Alginic acid also produces the same reaction as carageenin but this is the only other high-molecular weight compound known to have this effect. In reply to Dr. Glynn, Dr. Jackson said that it was not yet known whether alginic acid promotes the reabsorption of transformed collagen.

Dr. Gillman commented that in none of Dr. Jackson's slides had he seen evidence of fibrosis such as one usually sees in healing wounds. This indicated that the two phenomena, viz. fibrosis (such as scar formation) and Dr. Jackson's granuloma were quite different.

Dr. Banga said that extraction with dilute acetic acid and with citrate buffer of the same pH results in different material. This is suggested by the very great swelling of the fibres that occurs in the case of acetic acid extraction. Acetic acid dissolves a larger fraction of the fibres. Later Dr. Bowes said that the protein extracted with citrate buffer, pH 3.7, and acetic acid, pH 2.8, were different. With citrate buffer the protein extracted decreased in successive extracts; whereas with acetic acid there was no indication of such a decrease and it looked more as if the fibres as a whole were very slowly going into solution.

Dr. Jackson agreed that the property of the extract depended on the method of extraction.

In reply to Dr. Schwarz, Dr. Jackson said that the procedure for silvering the sections was based on the method of Lillie. Dr. Schwarz said that from the light microscope work alone it is impossible to say whether silver was deposited on the surface or was actually inside the fibrils. Dr. Jackson agreed but said that the fact that the ability to take up silver is lost without apparently altering the structure of the fibre indicated that the silver was on the surface.

Dr. Schwarz referred to electron-microscope work which showed that when interfibrillar cement was removed, for instance, by treatments with hyaluronidase, argyrophilia was also removed.

Dr. Robb-Smith said that injection of methyl cellulose produced a similar tissue effect to carageenin but that the methyl cellulose remains

and becomes encapsulated whereas the carageenin is absorbed and the tissue reaction is resolved.

Dr. Orekhovitch said that Dr. Jackson's data concerning the content in connective tissue of neutral salt solution-soluble, acid-soluble and insoluble collagens, after carageenin injection, does not justify the conclusion that the precursor of insoluble collagen is neutral salt-soluble collagen, rather than procollagen. The low content of procollagen during the first few days might perhaps be explained on the view that procollagen rapidly undergoes further transformations, as fast almost as it is formed. For this reason accumulation of procollagen in the tissues is not observed.

Dr. Neuberger said that the work of Dr. Gross on the physical properties of neutral extract collagen and the work of Dr. Jackson on granuloma and the work of Harkness *et al.*, indicated that, apart from a possible water-soluble fraction the neutral salt-soluble fraction must be regarded as a precursor of collagen. He suggested that insoluble collagen is not homogeneous but consists of a family of proteins of similar composition but having certain physical or chemical differences which lead to differences in solubility. There might, therefore, be no fundamental difference between acid-soluble and neutral salt-soluble collagen. There is at present no evidence to discriminate between this possibility and the possible conversion of neutral salt-soluble collagen through acid-soluble collagen into insoluble collagen.

Dr. Astbury said that in this work on carageenin there was the first evidence of a sulphated polysaccharide being directly concerned in the formation and aggregation of collagen *in vivo*, and asked if anything was known about the structure of carageenin. Dr. Jackson quoted work done in the National Research Council in Ottawa which showed that carageenin consists of two fractions, one a polygalactose and the other a polygalactose in which 30 per cent of the galactose was replaced by the anhydro form and also contained 30 per cent less sulphate. Dr. Meyer said that both fractions appeared to be branched polysaccharides and agreed with Dr. Astbury that they were not similar to polysaccharides which had been isolated from connective tissue. Dr. Gross suggested that it might be possible to distinguish between neutral salt-extracted collagen formed during the disintegration of collagen from that found during the formation of collagen by seeing whether it forms collagen fibrils on heating to 37° C. He had observed free hydroxyproline in extracts of collagenous tissue and suggested that this might be responsible for the observation of a water-soluble collagen but Dr. Jackson said that the hydroxyproline in his water-soluble collagen could not be removed by prolonged dialysis.

Dr. Baló referred to mucous granulomata in the lungs. These differ from those caused by carageenin in that they do not disappear after a time.

STRUCTURAL PROBLEMS ASSOCIATED WITH THE FORMATION OF COLLAGEN FIBRILS *IN VIVO*

SYLVIA FITTON JACKSON

An investigation of the elaboration of the fibrils of collagenous tissues involves three main issues; firstly, whether the collagen molecules and their precursors are synthesized solely within the cell and, if so, what specific organelles are concerned; secondly, how the collagen fibrils are formed from these precursors and whether the transformation is intra- or extracellular; thirdly, by what mechanism the collagen fibrils continue to enlarge, and form into bundles. The results described in this paper are concerned with these questions.

Studies on both living and fixed tendon and bone tissue of avian embryos have demonstrated the presence of many cytoplasmic granules in the collagen-forming cells, when intercellular material is about to be or is being deposited. Qualitative cytochemical methods have shown that the granules contain both protein and mucopolysaccharide; they appear to be capable of chemical synthesis for they also contain alkaline phosphatase and cytochrome oxidase. It has been postulated that the granules have fibrogenic properties and are concerned in the synthetic processes associated with the formation of the intercellular materials (Fitton Jackson, 1955). The cells are also strongly basophilic in most of their cytoplasm; this indicates the presence of ribonucleoprotein.

The differentiation of collagen fibrils is a morphological problem, and it has been followed by studying thin sections of developing avian tendon in the electron microscope. These observations on fibrogenesis in tendon tissue (Fitton Jackson, 1956a) show that filaments, of 80 Å in diameter, which are the first to be distinguished in 8-day embryos, differentiate into characteristic collagen fibrils. The development of the fibrous collagen seems to be closely correlated with the amount of extracellular space that is available; thus the first filaments are either within or intimately associated with the cytoplasm since there are no clearly defined intercellular regions. As age increases, however, intercellular areas appear (Fig. 1); they presumably contain interstitial fluid and they gradually become filled

with fibrils until finally bundles are formed. In longitudinal sections of 11-day embryonic tendon the fibril diameter is about 120 Å and the axial periodicity of immature collagen, viz. about 210 Å, is discernible, and agrees with that found in teased preparations taken directly from the same aged embryos. High angle X-ray diffraction of dried specimens of tendon from these embryos gives a recognizable fibre diagram for collagen (Fig. 2); the characteristic spacing of 2.86 Å is apparent and with increasing age the equatorial spot at 12 Å becomes more clearly defined.

Each collagen fibril within a bundle is surrounded by a structureless substance of low electron density; this has been termed interfibrillar material. Within the cross-section of the adult fibril a number of smaller units are sometimes visible, but their size and mode of packing is not, as yet, clearly defined (Fig. 3). Studies by X-ray diffraction (North, Cowan and Randall, 1955) have demonstrated that there is a large-scale lateral order corresponding to the grouping of protofibrils (composed of one or several polypeptide chains) into regularly arranged bundles. Thus it is tentatively suggested that the structural level of the units observed in the cross-section of adult fibrils may be intermediate between that of the collagen fibril and of the protofibrils.

Analyses of the development of avian tendon has provided quantitative data which show that the diameter of the collagen fibrils increases with age (Fig. 4) and that such enlargement is accompanied by a reduction in the relative amount of interfibrillar material which invests each fibril. The ratio of these substances may be defined briefly as the packing fraction of the fibrils within the bundles since it gives a measure of the degree of close packing of the fibrils and it has been plotted as a function of age (Fig. 4).

By the use of sections in which fibrils have been cut exactly transversely to the bundle axis it has been possible to investigate more accurately the relationship of these fibrils to the cells during the process of fibrogenesis. The morphological picture indicates that fibrils are formed both intra- and extracellularly. Firstly, groups of fibrils have been found well within the cytoplasmic areas (Fig. 5); these are ultimately extruded from the cell by a method that is not known. Secondly, fibrils arise outside the cell but often in close association with the cell surface; this method of elaboration predominates as age increases. During differentiation (Fig. 6) the cytoplasm of adjacent cells and the bundles of collagen fibrils are so

intimately related to each other that a jigsaw outline is obtained. If the cell surfaces are traced it becomes evident that the bundles are inter-connected by narrow channels of not more than 400 Å across so that the extracellular regions are continuous with each other. During development, it is noticeable that there are only small variations in the diameter of the individual fibrils at any one age

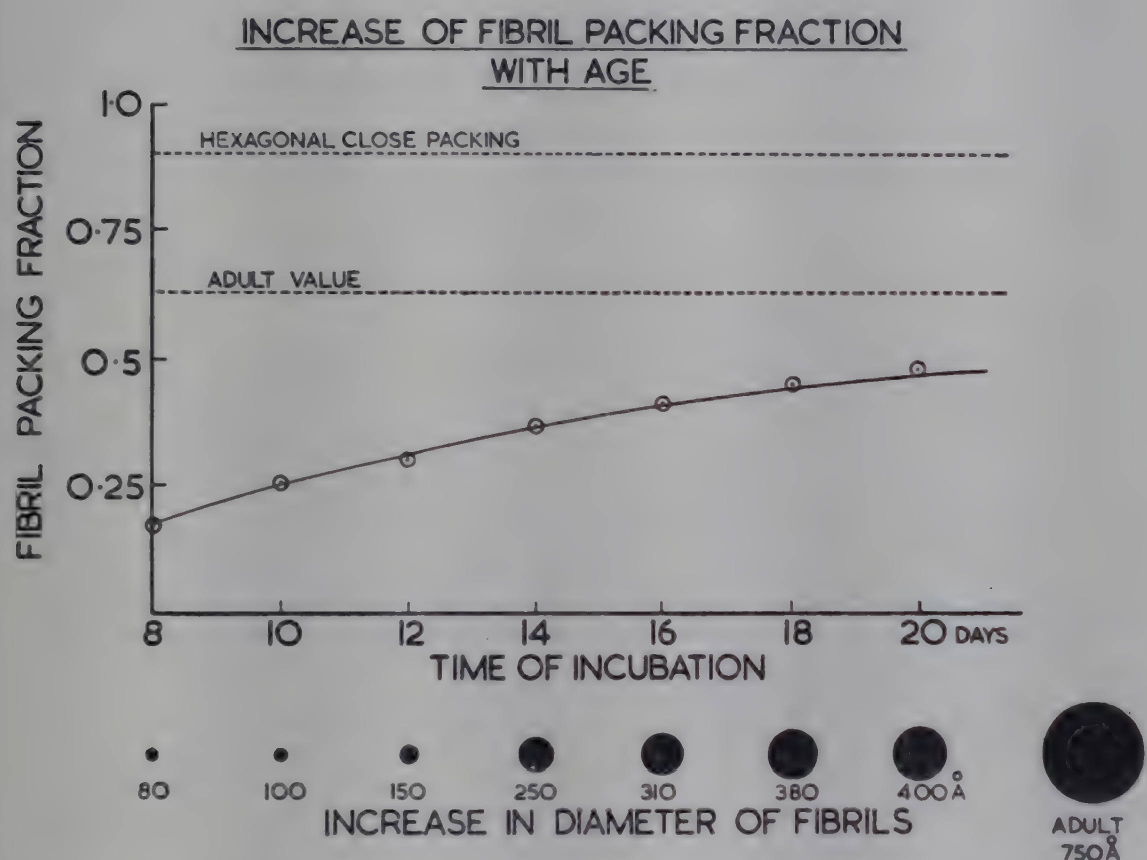


Fig. 4

Diagram of (*a*) the rate of increase in the diameter of collagen fibril with age and (*b*) the curve of the packing fraction of the fibrils in a bundle with age. The packing fraction of long solid cylinders is marked for comparison purposes as well as the figure for adult tendon (0.62).

which suggests that the fibrils must have been formed almost simultaneously or by a very strictly controlled mechanism.

As collagen-fibre bundles, detectable by light optical methods appear, a positive periodic acid-Schiff reaction is at first obtained from the intercellular regions; these areas also stain metachromatically, and the intensity of the stain increases as the regions enlarge. The intense metachromasia is then gradually lost and in the adult fowl it can only just be detected.

The formation of organized tissue involves various stages of differentiation. It is suggested that the first stage is one of chemical synthesis of the macromolecules; the second stage may be resolved by electron microscopy, and consists of the organization of the collagen and other molecules into fibrils and interfibrillar material; the final stage may be observed by light microscopy and includes the differentiation of the cells and intercellular material into the distinctive tissue.

In the present study, some evidence has been provided of probable synthesis within the fibrogenic cells of collagen protein or its precursor and of mucopolysaccharide and it is apparent that the cytoplasmic granules may be concerned in these synthetic actions. Thus it is believed that the first stage of differentiation may be represented by the formation of precursors and/or collagen molecules as a secretory product of the cells.

The method of the organization of the intercellular material as seen in thin sections of tissues suggests that it is formed when the cellular secretions come into contact with interstitial fluid and the transformation of the molecules into their fibrous form may thus be due solely to a change in environment, the interstitial fluid supplying, e.g. a medium of appropriate ionic strength and pH. This conversion may occur as a direct result of either the extrusion of secretions into the intercellular regions and subsequent reaction with the fluid, or, since Lewis (1941) has shown that the surrounding fluid may be introduced into the cell by pinocytosis, the cell secretions may come into contact with such fluid within the cytoplasm, and, after transformation would appear as an intracellular formation of fibrils. On the other hand, small groups of newly formed collagen fibrils may be taken into the cell by phagocytosis and thus appear to have an intracellular origin. It must also be taken into account that the transformation of the cellular precursors may not be due solely to change in environment, but that the interstitial fluid may provide additional organic and/or enzymatic substances essential for the final stages in the formation of the intercellular matter. The above hypothesis does not necessarily preclude other processes which may also be carried on simultaneously (e.g. Fitton Jackson and Randall, 1956).

The fact that the diameter and packing fraction of the fibrils have been shown to change with age has lead to the conclusion that the interfibrillar material must contain collagen molecules which, as

growth of the fibrils proceeds, are laid down in the form and packing appropriate to the fibre diagram for collagen protein. These results lend support to the hypothesis of Schmitt, Highberger and Gross, 1955, that the material extracted by neutral salts from mammalian skin is dispersed in the 'ground substance' of the tissue and is a precursor in physiological fibrogenesis; identical fractions also exhibit a fairly high glycine turnover rate (Harkness *et al.*, 1954).

In thin sections of tendon, the banded structure of the collagen fibrils is not clearly defined. In sections of early osteogenic tissue,

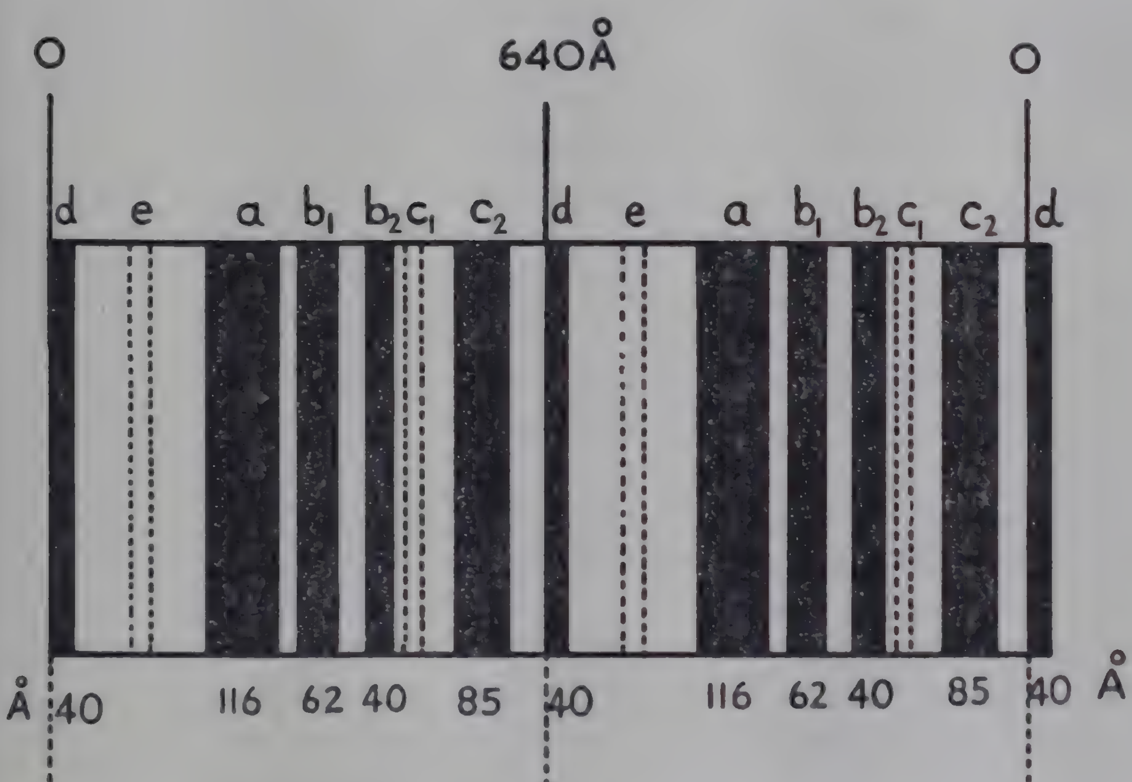


FIG. 7
Diagrammatic representation of the organization of the bands and interbands within two adjacent periods, and the corresponding width of the bands in collagen fibrils of periosteal bone.

however, the fibrils clearly show a mean periodicity of about 630 Å with a considerable degree of fine structure (Fitton Jackson, 1956b). The average width of the bands within a period (normalized to 640 Å) has been measured by means of densitometer traces of electron micrographs of newly formed fibrils, and is shown diagrammatically in Fig. 7. The diameter of the fibrils adjacent to the cells in periosteal bone is often about 400 Å, and transverse sections confirm that each fibril is invested by less dense interfibrillar material.

Fibrogenesis in bone-forming tissue is essentially similar to that in the tendon (Fitton Jackson and Randall, 1956).

At an early stage of calcification of bone, dense particles, of less than 100 Å in size, become localized in a ring almost exclusively between the *d* and *ab* bands (Fig. 8), i.e. within the major region of indentation of the main period of the fibrils. In transverse sections of the appropriate part of the fibrils, the particles are seen to be irregular in outline, are about 100 Å apart and number up to 10 per ring. Powder diffraction patterns have been obtained by electron diffraction from such sections, and measurements of the Bragg spacings are in close agreement with those obtained from a control specimen of hydroxyapatite. The particles must be related therefore to the deposition of the apatite content of bone. There is no clear evidence, however, from either electron microscopy or electron diffraction, of any preferred orientation of the particles with respect to the fibre axis, as has been demonstrated by means of X-ray diffraction by various authors, e.g. Engstrom and Zetterstrom (1951).

The localization of the apatite particles between the *d* and *ab* bands is precise; it may therefore be due solely to mechanical factors, and/or the attraction of specific chemical groups in the interband region. The differences in fine structure between the early ossifying and non-ossifying collagen fibrils suggest that the former may have some special morphological or chemical characteristic which may account for their ability to calcify.

These studies, however, have failed to demonstrate any notable feature which might indicate the method of formation of the striations of the collagen fibrils. It was hoped, therefore, that an investigation of fibrogenesis of other types of collagen might help to elucidate this problem. X-ray diffraction has identified collagen protein in the cuticle of earthworms (Astbury, 1945) but the characteristic axial periodicity of the constituent fibrils was apparently lacking (Reed and Rudall, 1948). It was thus of interest to try to determine if there were any features in the methods of fibrogenesis of banded, as opposed to unbanded collagen fibrils, which might account for the observed differences in the morphological structure of the fibrils, and hence deduce the method of elaboration of banded fibrils. The cuticle of *Lumbricus* sp. was therefore selected for study.

This work is still in a preliminary stage. Thin sections of the periphery of the worm show that the main body of the cuticle is composed of about 18 layers of unbanded fibrils about 1500 Å wide

and orientated in a criss-cross fashion at angles ranging from 74° to 106° to those of the adjacent layers. Transverse sections of the fibrils indicate that they are irregular in outline. The fibrils of the cuticle weave in and out of precisely arranged cytoplasmic processes which penetrate the cuticle from the underlying epidermal cells and which stretch between the cells and the outer membrane of the worm. The observations which have so far been made, suggest that the cytoplasmic processes may take part in the orientation of the unbanded fibrils for it is reasonable to assume that the epidermal cells secrete the precursors of the cuticle.

It has been found by cytochemical methods, that small granules are present in the cytoplasm of these underlying epidermal cells. These bodies are not the large mucinous globules, which are believed to be concerned in the production of the mucin which covers the exterior surface of the worm. The granules are periodic acid-Schiff positive, and may therefore contain polysaccharide. It is possible that they are homologous with the cytoplasmic granules in vertebrate collagen-forming cells. It is interesting that in their recent analysis of the earthworm cuticle (*Lumbricus* sp.) Watson and Smith (1956) have shown that its content of hydroxyproline is extraordinarily high, some 50 per cent more than the amount normally present in mammalian collagen, but it contains very little proline.

At the beginning of this paper it was stated that the problem of fibrogenesis involved three main questions and these questions have in part been answered. Firstly, some evidence has been provided which supports the view that the collagen protein molecules and their precursors are formed within the fibrogenic cell and that the cytoplasmic granules are concerned in the synthetic processes associated with the formation of the intercellular material. Secondly, it has been demonstrated that collagen fibrils arise both intra- and extracellularly but mainly the latter. Thirdly, it has been concluded that the interfibrillar material of collagen bundles in vertebrate tissues contains collagen molecules or their precursors, and that these are subsequently deposited on to the growing fibrils in the form and packing appropriate to the characteristic fibre diagram of the collagen protein.

GROUP DISCUSSION

Dr. Grassmann asked whether the distances of the various collagen sub-bands correlated with those he had described. Dr. Fitton Jackson

replied that in previous work there was, for instance, agreement in their measurements on rat-tail tendon but these measurements differed from those made on fowl-neck and kangaroo tendon. The osteogenic fibrils again show a difference in the width of the bands to those previously reported. Unstained, freshly teased fowl-neck tendon fibrils contained two E-bands whereas osteogenic fibrils contained only one E-band. There might thus be no precise correlation between bands of different tissues and species.

DR. GROSS noted the remarkably constant increase of collagen fibril diameter with age. Had Dr. Fitton Jackson found any bundles of collagen in older or adult preparations consisting entirely of small fibrils? DR. FITTON JACKSON said, no, she had not. She confirmed the extraordinary regularity in fibril growth. Hundreds of fibrils had been measured for each specimen in each age group, and the width did not vary beyond ten per cent either way. In fact, the age of the tissue could be gauged from measurements of fibril diameter in a given section of metatarsal tendon.

DR. ROBB-SMITH inquired whether the bundles of fibrils which appeared to be intracellular were not really extracellular being enclosed by cytoplasm of irregularly shaped cells, in other words 'invagination'. DR. FITTON JACKSON replied that although this might occur occasionally with single bundles the occurrence of groups of several, closely placed bundles made it unlikely.

DR. GILLMAN inquired as to the nature of the 'clear' areas surrounding sectioned fibrils in Dr. Fitton Jackson's lantern slides.

DR. FITTON JACKSON said the evidence indicated that the interfibrillar material contained collagen molecules or their precursors; it might also contain a small amount of polysaccharide, though no data was available at this point. The methacrylate had not been removed from the sections and therefore the original 68 per cent of water contained in the tissue should have been replaced by the methacrylate. The clear areas might have been increased by fibril shrinkage as compared with that existing in the material *in vivo* but shrinkage must have been of the same overall degree as otherwise she would not have obtained such a smooth curve from the ratio of the diameter of the fibrils to the interfibrillar material.

DR. HALL said he was interested in the first stage of differentiation described by Dr. Fitton Jackson and which she felt might be of a chemical nature. He pointed out that if one accepted the concept of the production of a primitive collagen, by the interaction of dissimilar helices, it would be at this stage that such a differentiation would occur. His own work gave evidence that this was so. In connective tissue brittle, anisotropic fibres with a high content of a polysaccharide indistinguishable from cellulose, and containing a protein fraction which on analysis proved to

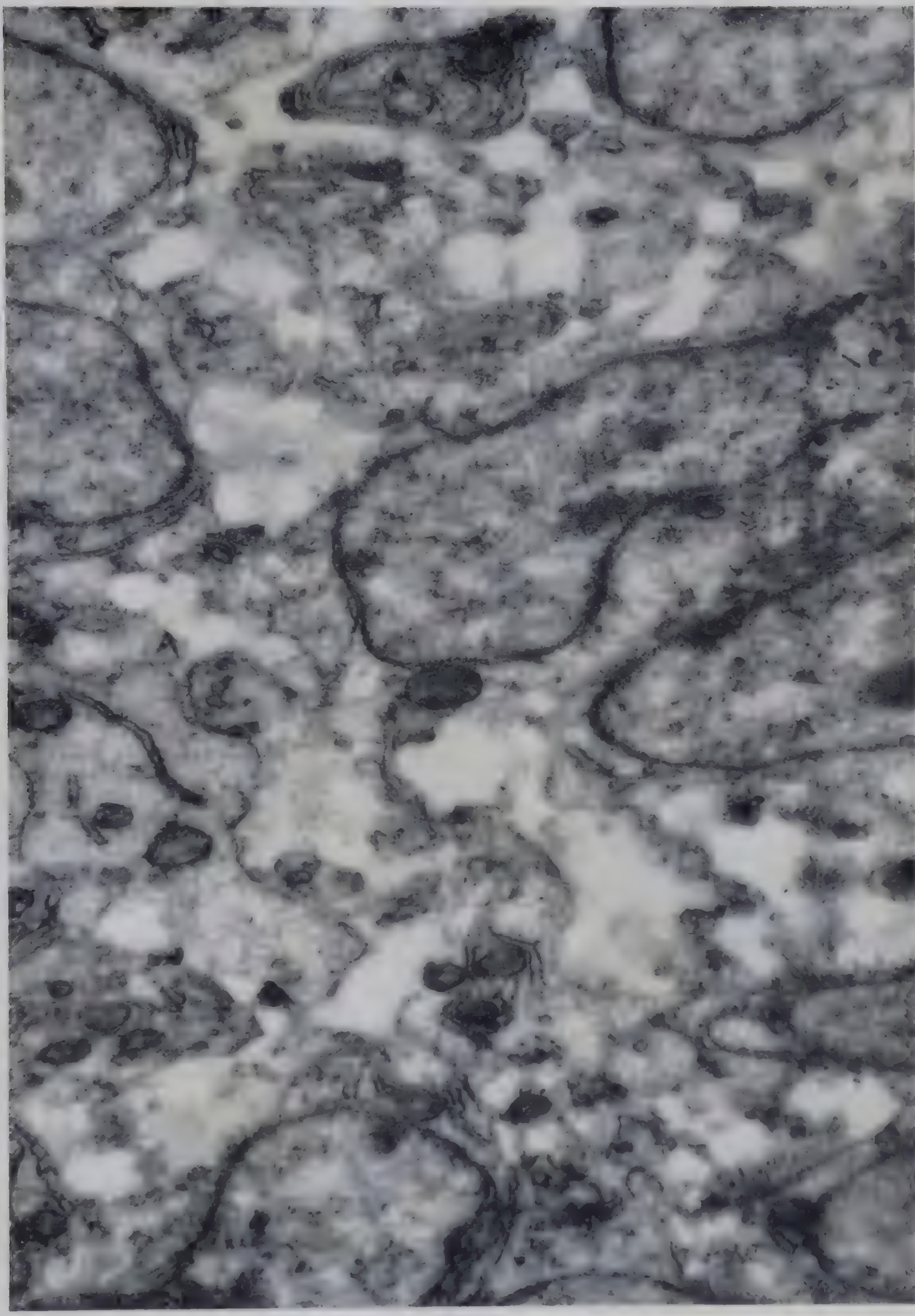


FIG. 1
Electron micrograph of a section of 10-day embryonic tendon; intercellular spaces have begun to appear between the fibroblasts and contain some filaments. $\times 28,000$.

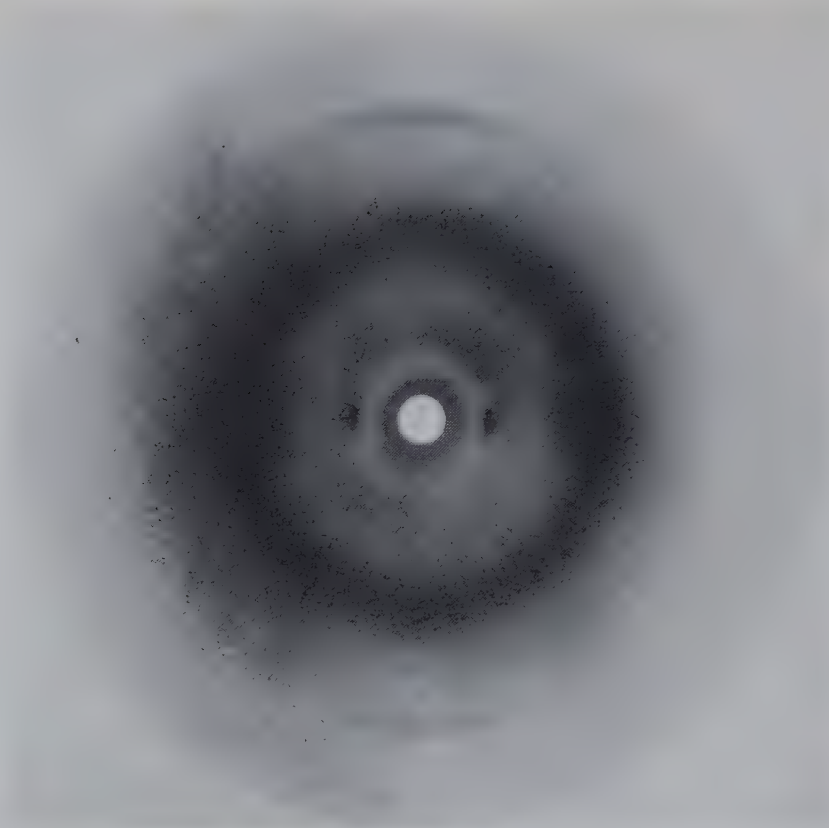


FIG. 2
High angle X-ray diffraction pattern obtained from the tendon of an 11-day embryo.

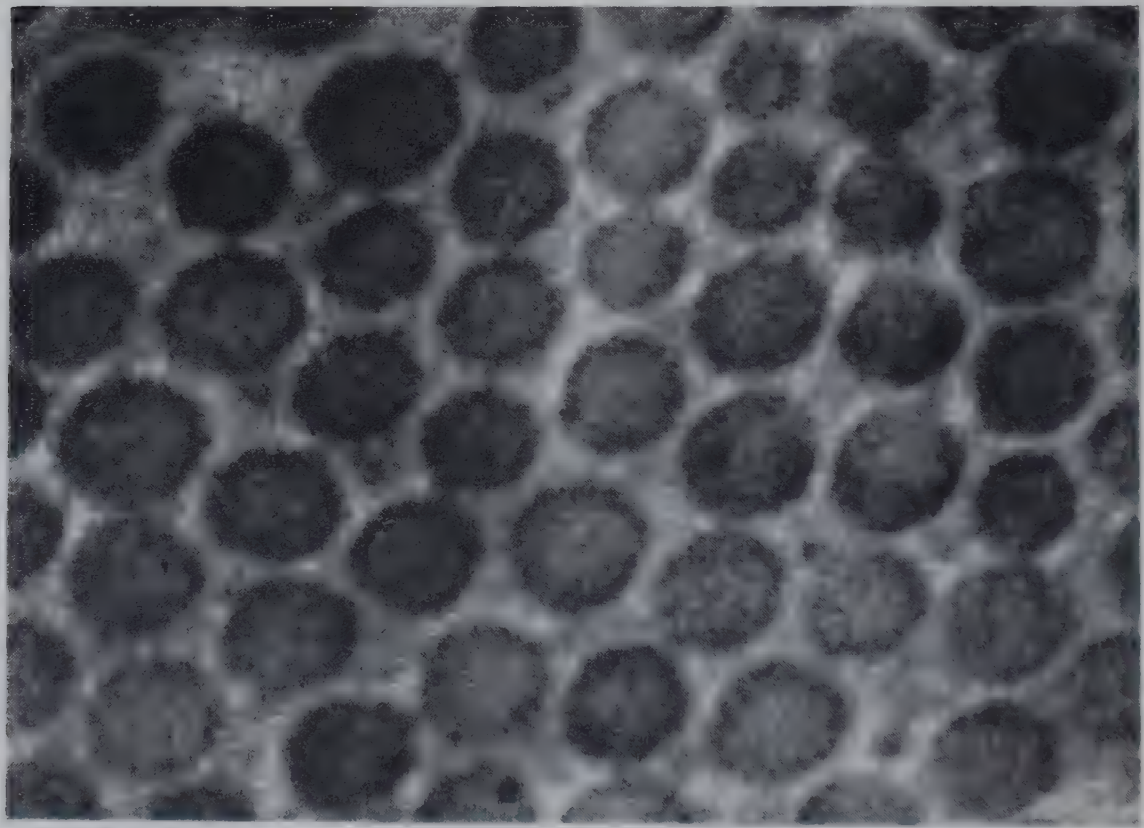


FIG. 3
Transverse section of tendon from an adult fowl. Each collagen fibril has a distinctive outer boundary and is invested by interfibrillar material. × 150,000.

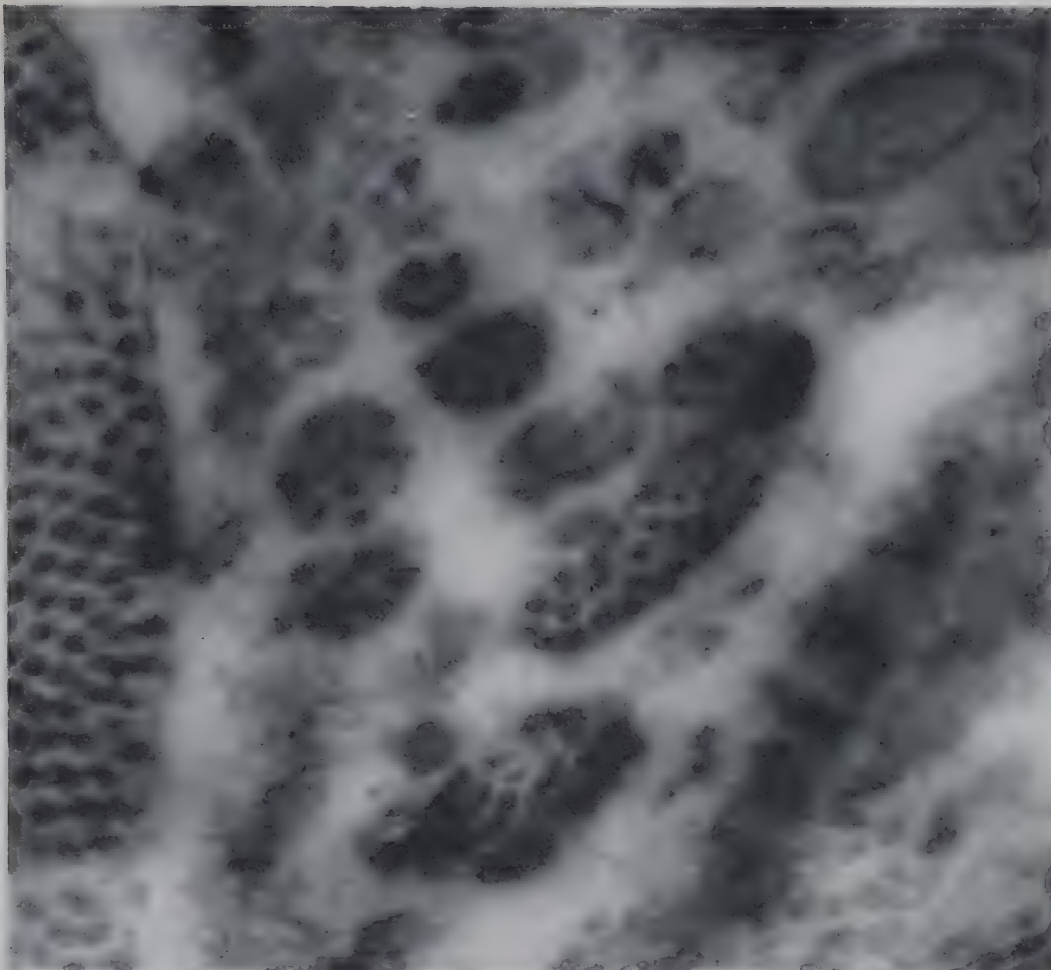


FIG. 5

In a 12-day embryo, small groups of fibrils are included within the cytoplasm of the cell and there is no evidence of adjacent cell surfaces surrounding the individual fibril groups. At the left of the micrograph, the cell surface is apparent. × 75,000.

FIG. 8

Section of periosteal bone. The well-formed structure of the collagen fibril is evident and dense particles are localized in one interband of each period.

FIG. 6
In an 18-day embryo, bundles of collagen fibrils are lying adjacent and in between two cells; the respective nuclei are very close to the bundles for there is not more than 200 Å between them, i.e. there is only a very thin sleeve of cytoplasm covering the nuclei. × 36,000.

be midway between collagen and elastin, could be demonstrated. This type of fibre was considerably increased under those conditions in which elastin-like structures could be produced from collagenous tissue by treatment with alkali. Thus it would appear that whereas under normal conditions, certain specific polysaccharides intervened at this stage of chemical differentiation to bring about the formation of collagen or elastin fibres from the precursors elaborated by the fibroblasts, it was possible for the wrong polysaccharide to react and become incorporated in the fibre with the production of a 'vitrified' structure.

Dr. Gustavson commented on the high hydroxyproline content (13 per cent) and the low proline content (1.5 per cent) of the earthworm cuticle, in spite of the 40° C. shrinkage temperature and temperature of dissolution reported by Rudall and Reed. Stabilizing hydrogen bonds involving hydroxyproline might be formed only when the molecules are oriented in a particular manner. The wide hydroxyproline-proline difference is unique.

Dr. Gross stated that the hydroxyproline-proline content of earthworm cuticle described by Dr. Jackson was confirmed independently by Highberger and by himself.

THE ACID MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE[1]

KARL MEYER, PHILIP HOFFMAN AND ALFRED LINKER

An interdisciplinary conference such as this one presents a unique opportunity to learn from other specialists problems and viewpoints which are different from those obtained in one's own narrow field of experience. In fact, it will undoubtedly take the combined and sustained efforts of the histologist, the physiologist and the chemist to assign both place and function to the bewildering array of acid mucopolysaccharides which have been isolated from various connective tissues and which show promise at growing more numerous in the coming years.

The following table lists the mucopolysaccharides which have

TABLE I

ACID MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

I.	Nonsulphated mucopolysaccharides
	1. Hyaluronic acid
	2. Chondroitin
II.	Sulphated mucopolysaccharides
	3. Chondroitin sulphate A
	4. Chondroitin sulphate B
	5. Chondroitin sulphate C
	6. Heparitin sulphate
	7. Keratosulphate

been identified with their characteristic properties and composition. (In this discussion only acidic mucopolysaccharides will be mentioned. Neutral or acid mucoids and glycoproteins which are present in connective tissues will not be considered since no defined compounds have been isolated nor have they been shown thus far to be distinct from plasma proteins.) The first group is sulphate-free and includes hyaluronic acid and chondroitin. There is some

[1] From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York, N.Y.

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evidence for an analogue of heparin which is either sulphate-free or undersulphated. The structure of hyaluronic acid has been fairly well established as an unbranched polymer of a disaccharide repeating unit, N-acetylhyalobiuronic acid, joined by β-4-0-glucosaminidic bonds. The structure of the disaccharide unit has been established as 3-0-(β-D-glucopyranosyluronic acid) 2-acetamido-2-deoxy-D-glucopyranose (Weissmann and Meyer, 1954). Hyaluronic acid, as isolated from different sources such as umbilical cord, synovial fluid, vitreous humor and some mesodermal tumours, does not appear to vary in its chemical properties, but does vary in molecular weight and degree of polydispersity. All samples of approximately the same degree of purity give identical yields of tetra- and disaccharides on exhaustive digestion with purified testicular hyaluronidase, and of a △ 4-5 unsaturated disaccharide on digestion with microbial hyaluronidase (Linker *et al.*, 1956). No evidence has yet been found for the occurrence in tissue of a *sulphated* hyaluronic acid or of hybrid saccharides of the type produced *in vitro* by the transglycosylative action of testicular hyaluronidase on mixtures of hyaluronic acid and chondroitin sulphates (Hoffman *et al.*, 1956). Hyaluronic acid probably occurs in most connective tissues although in greatly varying concentrations. It appears to be the product of the least differentiated fibroblast. In hyaline cartilage and in cornea, hyaluronic acid is probably absent.

Chondroitin appears to be an isomer of hyaluronic acid in which the D-glucosamine is replaced by D-galactosamine (Davidson and Meyer, 1954). Like hyaluronic acid, it forms viscous solutions, gives a mucin clot on acidification and is digested by both testicular and bacterial hyaluronidases at a rate comparable to that of hyaluronic acid. On acid hydrolysis, a good yield is obtained of the crystalline disaccharide chondrosine identical in its infra-red spectrum with chondrosine obtained from chondroitin sulphate A. (Its structure will be discussed below.) Chondroitin has only been isolated from cornea.

THE CHONDROITIN SULPHATES

On the basis of solubility, optical rotation and enzymatic hydrolysis, three chondroitin sulphates are distinguished which have been designated as A, B and C.

A and C appear to be closely related. On acid hydrolysis, they

both yield the identical crystalline disaccharide chondrosine, the deacetylated and desulphated repeating unit of the polymers. Both A and C are digested by testicular hyaluronidase to sulphated oligosaccharides, exhaustive digestion producing mainly tetrasaccharide. Chondrosine has been shown to be the galactosamine containing isomer of hyalobiuronic acid on the basis of the following scheme of reactions in Fig. 1. The first reaction shows that chondrosamine

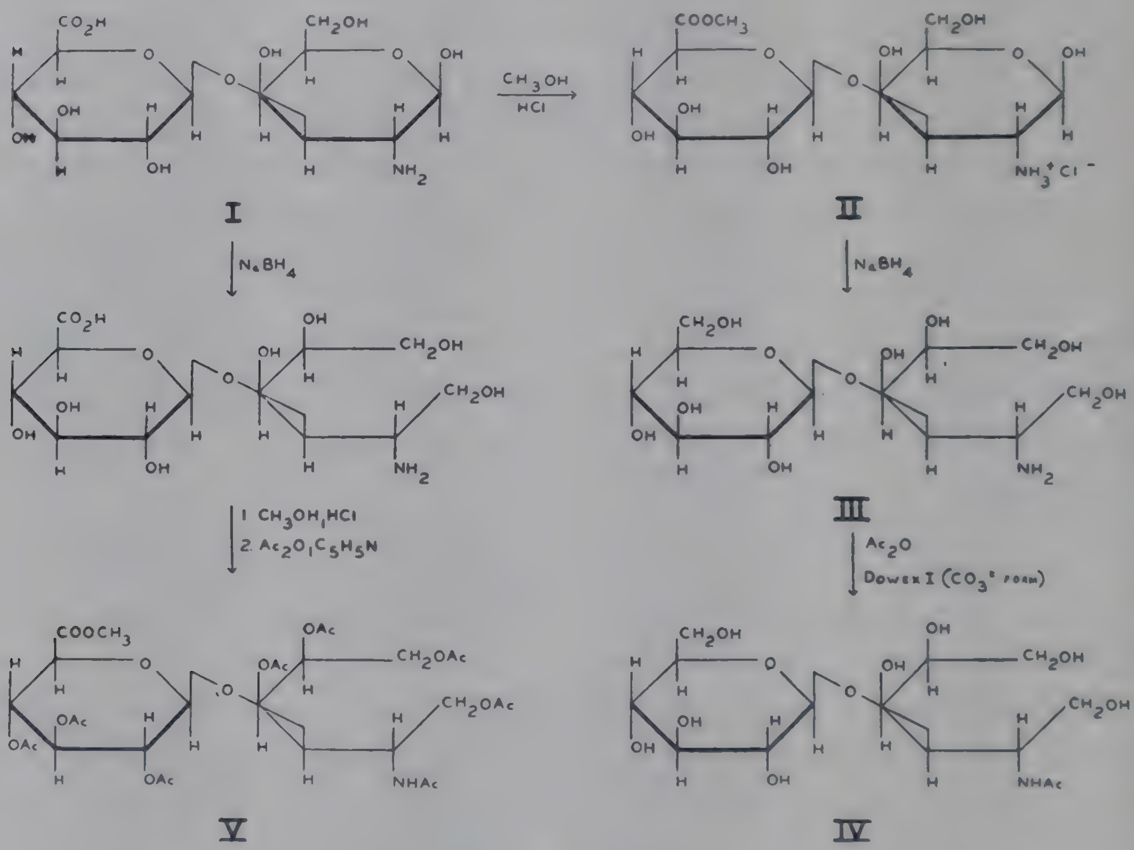

FIG. 1
Structure of chondrosine.

is on the reducing end. Chondrosinol obtained by borohydride reduction of the reducing end group was esterified and the resulting methyl ester was reduced to the alcohol. The resulting glucosido-chondrosaminol was resistant to α-, but was hydrolysed by β-glucosidase to yield glucose thus establishing the β-configuration of the glucuronidic bond. On acid hydrolysis of glucosido-N-acetyl chondrosaminol, D-glucose was obtained and isolated as the crystalline pentaacetate. This proves conclusively that the uronic acid in chondrosine is D-glucuronic acid. A 1-3 glucuronidic bond in chondrosine was demonstrated by periodate oxidation of a glucosyllyxitol obtained by deamination and subsequent reduction of

chondrosine methylester. The periodate consumption, resulting in the formation of 2 moles of formic acid and of one mole of formaldehyde, is only compatible with a 1-2 link of the glucosidolyxitol. Thus chondrosine was established as 3-0-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-galactose. The galactosaminidic bond is in all probability as in hyaluronic acid a β-1-4 linkage as evidenced from the formation of a 4-5 unsaturated disaccharide on hydrolysis with bacterial hyaluronidase of partly desulphated oligosaccharides obtained on partial acid hydrolysis of chondroitin sulphate (Davidson and Meyer, 1954; 1955). The sulphate group in both chondroitin sulphate A and C is in the galactosamine moiety. The tetrasaccharide obtained as the main product in exhaustive hydrolysis of the chondroitin sulphates (A and C) with testicular hyaluronidase, was digested with β-glucuronidase which cleaved the glucuronic acid moiety from the non-reducing end (Linker *et al.*, 1955). The resulting trisaccharide retained two sulphate groups. Whether the hydroxyl of carbon 6 or 4 is sulphated is not known. The most probable structure of chondroitin sulphate is presented in Fig. 2. The basis for the differ-

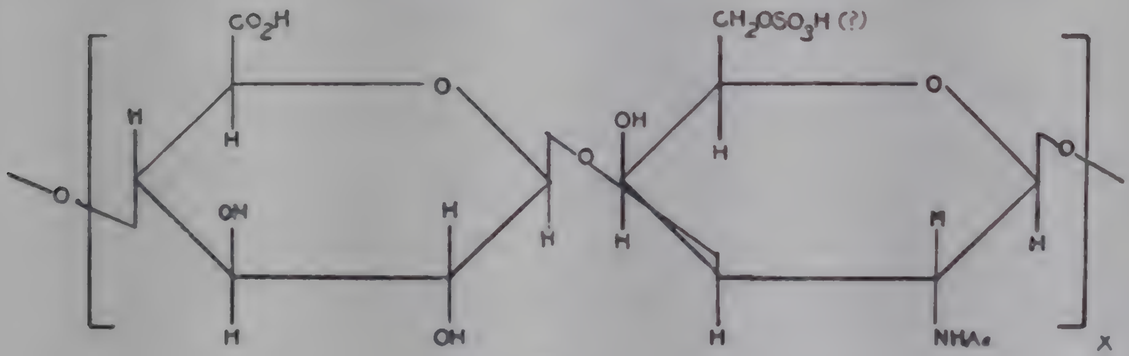

FIG. 2
Probable structure of chondroitin sulphate A.

ence in the properties of A and C is not known. From some tissues chondroitin sulphate fractions were obtained with marked sulphate deficiencies. They were especially abundant in growing (calf) bone. The sulphate deficiency does not appear to be due to loss of sulphate during the preparation. The sulphate deficient fractions are hydrolysed by bacterial hyaluronidases with the production of unsaturated uronides. As in model experiments the enzymes attack the hexosaminidic groups only where the sulphate is missing. Whether the incompletely sulphated fractions belong to the A or C series or to both cannot be decided at present.

Chondroitin sulphate A has been demonstrated in cartilage, bone,

cornea, aorta and in ligamentum nuchae and in chondrosarcoma. C has been isolated from cartilage, umbilical cord and other primitive connective tissues, tendon, nucleus pulposus (Ott, 1954), a human chordoma and an osteosarcoma.

Chondroitin sulphate B, like A and C, is composed of equimolar concentrations of N-acetyl D-galactosamine, uronic acid and sulphate. It has an optical rotation of about −60° and a lower solubility (as Ca salt) than either A or C and in contrast to the latter is completely resistant to hydrolysis by testicular or bacterial hyaluronidase (Meyer and Rapport, 1951). This resistance even persists with the partly or completely desulphated compound. On decarboxylation, it yields one equivalent of CO_2 per mole of hexosamine while in the carbazole method, which gives one equivalent with A and C, it gives approximately 50 per cent of the uronic acid value. On graded acid hydrolysis, B yields two uronido-chondrosamines differing in mobility on paper and in colour values, and two distinct hexuronic acids (Meyer *et al.*, 1956) (Fig. 3). One of these appears to be chromato-

STERIC RELATIONSHIP BETWEEN GLUCURONIC, IDURONIC, MANNURONIC AND GULURONIC ACID

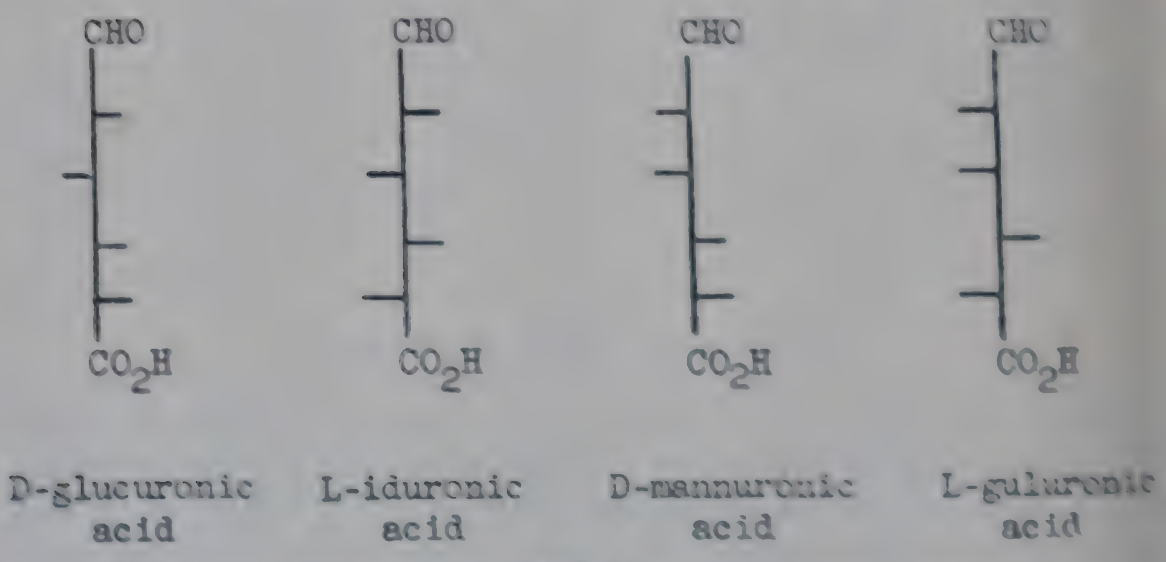

Fig. 3
Steric relationship between glucuronic, iduronic, mannuronic and guluronic acid.

graphically identical with D-glucuronic acid, the other with L-iduronic acid, the 5-epimer of glucuronic acid as the free acid and as lactone, in a variety of solvents. It is of interest that L-iduronic acid is the 5-epimer of D-glucuronic acid (the 5-epimer of D-mannuronic acid, L-guluronic acid, has recently been demonstrated in alginic acid

and in a variety of brown algae in which guluronic and mannuronic acid occurs in varying proportions (Fischer and Dörfel, 1955). On the basis of the occurrence of both glucuronic and iduronic acid in chondroitin sulphate B, attempts were made to ascertain whether or not a mixture of polysaccharides was present. These have been inconclusive. The chemical and physical properties of B, obtained from a variety of sources and by different methods, were closely similar. Such fractions have been isolated from skin, tendon, heart valves, *ligamentum nuchae* and aorta. 'β-heparin' obtained from beef lung (Marbet and Winterstein, 1951) is identical with chondroitin sulphate B as is a fraction obtained by Smith and Gallop (1953) from hog stomach. However, the possibility still exists that chondroitin sulphate B is a mixture of at least two hyaluronidase-resistant polysaccharides.

KERATOSULPHATE

This is the only sulphated mucopolysaccharide free of uronic acid. It is composed of equimolar amounts of N-acetylglucosamine, galactose and sulphate (Meyer *et al.*, 1953). Its structure is unknown and no glycosidase has been found which attacks this polysaccharide. The desulphated polymer,[1] however, is hydrolysed by an enzyme obtained from *lactobacillus bifidus* with the production of monosaccharides. It is of some interest that the sulphated polymer does not cross react with blood group substances or with type XIV *pneumococcus* antiserum. The desulphated polymer precipitates type XIV *pneumococcus* antiserum comparable to group A substance of hog gastric mucosa. (We thank Dr. E. Kabat for these data.) Keratosulphate has been isolated from cornea, where it represents about 50 per cent of the total mucopolysaccharide and from growing (calf) bone, where it is only a minor component.

FRACTIONS RELATED TO HEPARIN (HEPARITIN SULPHATE)

Mucopolysaccharide fractions of a strong positive rotation and composed of equimolar amounts of glucosamine, uronic acid

[1] We are greatly indebted to Dr. Maxwell Schubert for the communication to us of a method of desulphation of chondroitin sulphate A. The method yields desulphated high polymers in good yield with chondroitin sulphate A, B, C and keratosulphate. The complete desulphation of heparin has presented additional problems. The desulphated products are proving extremely useful in structural studies.

and sulphate and resistant to testicular hyaluronidase have been isolated from bovine aorta and from human amyloid tissue. Like heparin, these fractions exhibit high carbazole and low orcinol values relative to their hexosamine content. Some of these fractions are hydrolysed by a heparinase extract obtained from a flavobacterium (Paysa and Korn, 1956). The structure of these mucopolysaccharides is unknown.

Of the tissues studied by our group, four will be discussed in detail, namely, tendon, skin, *ligamentum nuchae* and aorta, primarily because these are the tissues most frequently mentioned in the abstracts of this symposium.

In the Achilles tendon of calf and pig, acid polysaccharide content by isolation was approximately 0.5 per cent by weight of the acetone and ether defatted and dried tissues. The polysaccharide was fractionated into three components, chondroitin sulphate B and C, present in about equal quantities, and hyaluronic acid present as a minor constituent. A similar distribution was found in heart valves.

In adult pig skin, we again isolated the same three polysaccharides, but in quite different distribution. Here chondroitin sulphate B was the major component followed by hyaluronic acid while chondroitin sulphate C was isolated in very low yield. (There is at least another not yet identified acid polysaccharide present.) In pig embryo skin (mostly at term) the total polysaccharide was very much higher than in adult skin. But here the main polysaccharide was hyaluronic acid. The ratio of chondroitin sulphate B to hyaluronic acid in adult skin was 1.25, in embryo skin 0.20. The distribution of chondroitin sulphate and hyaluronate was investigated in split oxhide (obtained through the courtesy of Dr. E. F. Mellon of the U.S. Dept. of Agriculture, Eastern Utilization Research Branch, Philadelphia). Three sections were obtained, a grain, a middle and a flesh layer. The grain layer contained almost all the elastic tissue. Table II gives the total yields and the ratio of chondroitin sulphate to hyaluronate.

The ratio in the total skin is similar to that of pig skin. The concentration of chondroitin sulphate B is practically constant in the three layers while the hyaluronic acid concentration in the grain layer was almost twice that in the other layers. According to data furnished by Dr. Mellon, the collagen cantent does not differ greatly between the grain and the corium layers, while the elastin content is approximately six times higher in the grain than in the corium layers.

TABLE II

DISTRIBUTION OF MUCOPOLYSACCHARIDES IN SKIN

Sample	*Polysaccharide (mg. per cent)* Total	CH.S. B	Hyaluronate	Ratio
Oxhide grain cut	410	177	226	0.79
,, middle cut	285	183	102	1.8
,, flesh cut	340	208	115	1.78
Combined total oxhide				1.29
,, ,, pigskin				1.25
,, ,, pigskin embryo				0.20

Bovine ligamentum nuchae like skin contains chondroitin sulphate B and hyaluronate as major mucopolysaccharides, ratio chondroitin sulphate B/hyaluronic acid = 1.5. But a third component in this tissue appears to be chondroitin sulphate A. None of these fractions was hydrolysed by crude elastase of pancreas or of mould origin (obtained through the courtesy of Dr. A. Bloch).

A pattern of mucopolysaccharide remarkably different from that of *ligamentum nuchae* was found in abdominal aorta, both bovine and human. From abdominal aorta of young (1-2 year) cattle as in *ligamentum nuchae*, hyaluronic acid, chondroitin sulphate B and A were isolated. In addition, there appeared in aorta a new fraction

TABLE III

DISTRIBUTION OF MUCOPOLYSACCHARIDES IN BOVINE LIGAMENTUM NUCHAE, BOVINE ABDOMINAL AORTA, AND HUMAN ABDOMINAL AORTA

	CH.S. B	*Hyaluronate*	*CH.S. A*	*Heparitin sulphate (mg. per cent)*
Bovine ligamentum nuchae	~225	~110	~110	—
Bovine abdominal aorta	~130	~230	~450	~130
Human abdominal aorta	~100	<100(?)	~400	~100

heparitin sulphate (see above, Table III). The total polysaccharide was approximately 1 per cent for cattle and 0.8 per cent for human aorta of the acetone and ether defatted and dried tissue. In bovine aorta, approximately 45 per cent was chondroitin sulphate A, followed by hyaluronic acid (23 per cent), while chondroitin sulphate B and hepari-

tin sulphate combined represent the rest in approximately equal concentrations. The human aortae were of persons with moderate atherosclerosis of 45 to 84 years (average 64.1) and yielded the same polysaccharides as young bovine aorta and in strikingly similar concentrations, with the exception of hyaluronic acid which, if present at all, was well below 10 per cent of the total. The diminution of hyaluronic acid with age has also been observed in adult versus embryonal skin. It is obvious from the data on skin, *ligamentum nuchae*, and aorta that chondroitin sulphate B is not a constant component of elastic tissue but that all three chondroitin sulphates are components of collagenous tissue. The histological interpretation of the differences in type and distribution of these sulphated polysaccharides in the various tissues appears, however, not possible at present.

SUMMARY

The acid mucopolysaccharides isolated from various sources have been discussed. The chemical structure of most of these is still unknown. Remarkable differences exist both in the types and in the distribution patterns of the acid polysaccharides in the different types of connective tissue.

GROUP DISCUSSION

DR. ASBOE-HANSEN asked if heparin had been detected in skin.

DR. MEYER replied that in his early experiments some anti-coagulant activity had been observed in extracts of pigs' skin. However, when CSA/B was carefully purified it had no heparin activity. CSA/B is identical with the beta-heparin of Winterstein which had been reported to have high anti-coagulant activity.

In answer to a question from Dr. Hall, DR. MEYER said that no polysaccharide components other than those that he had described had been detected in *ligamentum nuchae*. Moreover, he had not been able to demonstrate any mucolytic action in crude preparation of elastase. CSA/B and CSA/A were prepared from *ligamentum nuchae* by alcohol fractionation of their calcium salts. In other tissues, for example calf bone, CSA/A and CSA/B could only be separated if the mixture of mucopolysaccharides is first of all treated with hyaluronidase.

DR. GILLMAN asked if one might anticipate differences in the micro-

scopic and sub-microscopic organization of elastic fibres from aorta and from skin, in view of the differences in the chondroitin sulphate and mucopolysaccharide contents of these two tissues noted by Dr. Meyer. Dr. Reed said that the morphology of elastin fibres from the skin is very similar to that of elastin fibres from the aorta.

Dr. Robb-Smith wondered whether the high content of CSA/A in aorta and cartilage could be correlated with the vascular and skeletal changes in experimental lathyrism and whether experimental work with beta-proprionitrile might throw some light on their metabolic behaviour.

Dr. Meyer did not know but said that CSA/A also occurs in cornea in which morphological or clinical changes in lathyrism have not been described. However, CSA/A constitutes a much greater fraction of the polysaccharides in aorta than in cornea. In answer to Dr. Gillman, he said he had done no work on polysaccharides in the lens.

In answer to Dr. Sylvén, Dr. Meyer said he did not know whether heparitine sulphate inhibited the clotting reaction of heparin but that CSA/B does have this effect. He was referring here to work of Dorfman *et al.* (*Fed. Proc.*, 1956).

Dr. D. S. Jackson asked what Dr. Meyer's opinion was on the possibility of linkages between protein and polysaccharides in connective tissue.

Dr. Meyer said that any bonds between hyaluronic acid and protein are comparatively weak. Hyaluronic acid can be prepared chemically free of protein, and the physical properties of the material are almost identical with those of a hyaluronic acid protein complex prepared by Ogston, which contained about 30 per cent protein. This does not apply to hyaluronic acid from all tissues. For example, it applies to hyaluronic acid from umbilical cord and streptococci but not to material isolated from synovial fluid. Sulphated polysaccharides on the other hand are present in the tissue as protein complexes. The work of Schubert has demonstrated this with CSA. The native complex and complexes prepared by adding protein to purified CSA differ in their physical properties.

Dr. Meyer also had evidence for a stable protein polysaccharide complex in heart valves. The complex migrates electrophoretically as one component and cannot be dissociated by salt solution or by raising the pH to 9-10. The protein is rich in tyrosine and tryptophan. The type of bond between protein and polysaccharide is obscure.

Dr. D. S. Jackson referred to the finding of Dr. Helen Muir that the viscosity of CSA is greatly reduced by treatment with papain to digest the protein fraction. He also reported that a gelatinous fluid obtained from Heberden's nodes contained only hyaluronic acid and no protein-free or bound to the hyaluronic acid. Dr. Partridge said he had repeated

Schubert's work. After removal of collagen by means of an ion-exchange resin he obtained a mixture of free CSA and a complex of CSA and protein. This complex could only be split by hydrolysis with alkali or acid and the amino-acid analysis of the protein fraction was found to be quite different from that of collagen.

Dr. Neuberger and Dr. Meyer discussed possible reasons for the glycol grouping in hyaluronic acid being unreactive to periodate. This is probably not due to the trans-configuration of the glycol grouping as this also occurs in glycogen. It also seems unlikely that it is due to hydrogen bond formation or to lactone bond formation. The former is unlikely for steric reasons, the latter is unlikely since most of the carboxyl groups can be esterified. The most probable reason seems to be inaccessibility of the grouping.

Dr. Hall reported an observation by Dr. P. F. Lloyd of Bangor that the electrophoretic mobility on paper of CSA/B prepared from *ligamentum nuchae* is increased by about 10 per cent after treatment with testicular hyaluronidase.

Dr. Meyer could not suggest an explanation for this.

EVALUATION OF EXTRACTION METHODS FOR ACID TISSUE POLYSACCHARIDES

Olle Snellman

The chemistry and physiology of the acid polysaccharides of animal origin (containing amino sugar and hexuronic acid) attract interest in many fields of biochemical and biological research. Differentiation between various types of chondroitin sulphuric acids has been performed during the last years (Meyer and Rapport, 1951) and several methods of isolation have been described. The polysaccharides are, however, difficult to isolate and separate since the amounts occurring often are small and the different polysaccharides show similarities in structure and, from many tissues, they are even obtained as a mixture difficult to separate.

For most workers in this field, the main goal has been to prepare pure substances in order to perform structural determinations on the bonds between the different monosaccharides building up the polysaccharides. Little attention has been paid to the quantitative aspects and few attempts have been made to obtain the acids in a 'native' state, i.e. in an, as far as possible, undegraded state.

Besides the importance of getting a clear picture of the bonds and structure of the different polysaccharides, the latter questions deserve much attention. We have to know something about the state and the quantitative aspects of the different polysaccharides in the tissue since in diseases of connective tissue, commonly termed 'collagen diseases', the earliest and most profound alterations are found in the ground substance. It is with these aspects in mind we have made an investigation of the extraction methods of the acid polysaccharides.

The quantitative extraction of the acid polysaccharides is a matter of considerable difficulty and dilute sodium hydroxide is still frequently employed. This method was investigated by Jorpes (1929) using cartilage to obtain chondroitin sulphuric acid. He showed that a concentration as high as 0.5 N was necessary to extract the acid polysaccharides from cartilage at 0° C. However, the polysaccharides were obtained in a degraded state.

Many investigators have used the alkaline extraction procedure, e.g. Levene and Forge (1914), Fürth and Bruno (1937), Karrer and

Mayer (1937), Bray, Gregory and Stacey (1944), Meyer, Odier and Siegrist (1948), Strandberg (1950) and Leon and Deiss (1954).

Meyer and Smyth (1937) suggested the use of strong solutions of calcium chloride and obtained good yields of chondroitin sulphate by the extraction of dried cartilage powder with 10 per cent calcium chloride solution at neutral reaction. Blix and Snellman (1945) used calcium chloride extraction and were careful to avoid acid or alkaline solutions during the subsequent separation procedures. The aim was to obtain, as nearly as possible, the polysaccharides in an undegraded state.

Einbinder and Schubert (1950) stored the cartilage in the cold for several weeks and could then extract chondroitin sulphate with 30 per cent potassium chloride at pH 7-8 in good yields, thus using a very mild method. Malmgren and Sylvén (1952) showed that from 19.5 g. dry weight of bovine nuclei pulposis, 3.2 g. could be extracted with this method and, by subsequent extractions using alkaline solutions, 0.80 g. could be removed. The tissue residues after this extraction were, however, not investigated.

Hyaluronic acid generally occurs together with sulphated polysaccharides. The extraction procedures of this acid vary somewhat among different workers but, principally, the methods are quite similar. Most of the principles used originate from the work of K. Meyer, the first to isolate hyaluronic acid.

Fluids such as synovial fluid, tumour fluid and vitreous humor are generally treated with acetone or acetic acid to precipitate the hyaluronic acid and protein as an associated mixture or complex. Sometimes the protein content is even increased by addition of serum prior to the precipitation in order to increase the amount of polysaccharide precipitated in a mucin-like clot. From the clot the hyaluronic acid is taken up by extraction with potassium acetate at pH 9. Proteins are then removed from the solution by shaking with chloroform-amyl alcohol (Sevag, 1934) and further contaminations are removed by Lloyd's reagent at pH 4.

Meyer (1948) prepared sodium hyaluronate of high viscosity by extracting homogenized human umbilical cords with 2 per cent phenol and clotting the diluted mixture with acetic acid. The proteins were then removed avoiding a pH greater than 7.5 in each step.

Dorfman and Ott (1948), Alburn and Williams (1950) and Jeanloz and Forchielli (1950) released hyaluronic acid from finely ground cords by digestion with pepsin at pH 2 for 24 hours at 37° C. and

then under similar conditions with trypsin at pH 7.4. After filtration the proteins have been eliminated from the solution by shaking with chloroform-amyl alcohol.

In order to isolate hyaluronic acid and chondroitin sulphuric acid from skin, alkaline solutions have been widely used (Meyer and Chaffee (1941), Pearce and Watson (1949), Schiller, Mathews *et al.* (1954)). It seems that some linkages between the non-denaturated proteins and the mucopolysaccharides (lysine?) contribute to the difficulties in the separation of protein and polysaccharide when alkaline solutions are not used.

Schiller *et al.* (1954) treated the skin with a 2 per cent alkaline solution for 24 hours. They neutralized and dialysed the solution and incubated with trypsin for 5 days. Afterwards, they precipitated with trichloroacetic acid, centrifuged and dialysed again. The solution was precipitated with ethanol in the presence of 1 per cent potassium acetate. On the precipitate, a slab electrophoresis was made according to Gardell *et al.* (1950). Hereby they could separate the hyaluronic acid and chondroitin sulphuric acid.

Gardell (1952) has boiled the tissue and afterwards digested it with an extract of pancreas for many days and purified the product with 10 volumes of ethanol and later with Lloyd's reagent. A slab electrophoresis was then performed.

In the case of heparin, the method described by Charles and Scott (1933) appears to have been generally adopted. Here the tissue is first autolysed for 24 hours at room temperature, since higher yields are then obtained. The tissue is extracted at 50° C. for one hour with a solution consisting of 62 parts of 0.5 N sodium hydroxide and 8 parts of saturated ammonium sulphate solution. After heating rapidly to 70° C., the mixture is filtered and adjusted to pH 2.5 to precipitate the heparin-protein complex. The precipitate is washed by different means and then dissolved in alkaline solution, and the proteins are subjected to trypsin digestion.

The very crude heparin obtained is further purified by a lengthy procedure which differs with different investigators but can cause heavy losses. For a critical evaluation of the different processes see Homan and Lens (1948), who have worked out a tedious procedure, which is said to cause not so heavy losses.

In this short description we have only reviewed the main principles used to extract the three groups of acid polysaccharides. Some methods giving low yields have been omitted.

Besides the various extraction methods, quite another method has been used by Consden *et al.* (1953) to obtain knowledge about the saccharides occurring in the tissue. Consden autoclaves smaller pieces of the tissue in water in order to remove the collagen and then he hydrolyses and determines the different monosaccharides in the hydrolysate. This method can give valuable information about the basic carbohydrates that occur in the tissue but the method has only a limited value since it does not give any informations about the polysaccharides to which the carbohydrates belong.

It may be appropriate to give some comments on the different methods before discussing the method we have worked out recently. At first, it is very difficult to judge how quantitatively the different methods extract the polysaccharides as we are lacking careful analyses on that point. Furthermore, all investigators extracting hyaluronic acid and chondroitin sulphuric acid have completely missed the heparin. It seems either to have been lost or obtained as a contamination in the fraction of the other polysaccharides, difficult to separate from them. Thus Sylvén and Malmgren (1952) purify hyaluronate by precipitating sulphate-containing polysaccharides with Azure A. Apparently, many authors have not succeeded in preparing sulphate free hyaluronic acid.

In a great number of investigations, alkaline solutions have been used to dissolve the tissue. I have the impression that the best yields are obtained by this method, and if any losses are obtained, they mostly come in the subsequent purifying processes.

However, the use of alkaline solutions involves the danger of degrading effects on the polysaccharides, and when this method is used nothing can be said about the 'native' state. The experience from other high molecular weight substances, as well as from hyaluronic acid, shows that high pH ought to be avoided.

The methods used for umbilical cord and other particularly easily extracted tissues do not have these disadvantages, at least to any high degree. But they are not quantitative and mixtures have been obtained difficult to separate. Thus, Hadidian and Pirie (1948) report in their fractions of hyaluronic acid, sulphur contents of 0.5–1 per cent, as usual. Most investigators have only, as it seems, been interested in a fairly good yield of hyaluronate not looking for other polysaccharides occurring.

The method of Einbinder and Schubert is also a mild method and may give an undegraded product. It is worked out for cartilage and,

to obtain good yields, the material is stored for a long time. What this treatment does to the product we don't know. Evidently according to the investigations of Malmgren and Sylvén (1952), the method is not quantitative.

Nearly every investigator has used his own purification scheme, often only a modification of some principles used in all the methods.

Many have used precipitation reactions going from very alkaline solutions to very acid solutions. These methods involve considerable danger of losses and danger of destroying part of the polysaccharides through breakdown effects. It seems that all such methods ought to be avoided as far as quantitative and 'native' aspects are concerned.

Kaolin has been used in several cases as an adsorbent but the proteins are not effectively removed by this mean and, if the extracts are too heavily treated by kaolin, losses of polysaccharide will occur. Nor is phenol or glacial acetic acid effective.

The method of shaking the extract with chloroform-amyl alcohol has often been used in order to separate protein and polysaccharide. The method involves a denaturation of proteins. If a quantitative separation is wanted, the treatment must be performed for a very long time, when used directly on the extract. Much of the polysaccharides, together with proteins, go into the organic phase or are situated in a middle layer and will only slowly be released, going back again into the water phase. Some contaminations remain in the solution. It seems better to purify afterwards with $Zn(OH)_2$ than by Lloyd's reagent.

Concerning the two types of polysaccharides hyaluronic acid and chondroitin sulphuric acid, the best method to release them from their protein associations seems to be by proteolytic enzyme digestion. The fission products are then comparatively easily removed.

Of the proteolytic enzymes used, pepsin has too low a pH optimum. Crude pepsin is also, according to Meyer (1945), often contaminated with a polysaccharide which might render this treatment dangerous. Crystalline trypsin or chymotrypsin can be used, but are said to be less effective (Gardell, 1952). Gardell uses a crude glycerol extract from the pancreas and the intestine. This is, however, contaminated by polysaccharides and contains at least one enzyme which splits glycogen. Crystalline papain might be used but it has not been tried. We have used a purified ficin which has a suitable pH optimum and is free of carbohydrates.

After the enzyme digestion, a preliminary purification can be achieved by dialysis and precipitation by organic solvents.

In many investigations the separation of hyaluronic acid and chondroitin sulphuric acid has been performed by precipitation of the calcium salts with ethanol. Precipitation with a 20 per cent ethanol solution is said to separate the polysaccharides. In spite of much use, the method has not been thoroughly investigated and, in the rough manner it has been used, no very good separations have been achieved. Thus Meyer and Chaffee (1941) use pneumococcal hyaluronidase in order to purify the chondroitin sulphuric acid further. Any kind of electrophoretic separation, as used by Gardell and Schiller *et al.*, is superior.

After these comments I will discuss some points in our own extraction method (Snellman and Ottoson) which is going to be published shortly. The method has hitherto been used mainly on skin, since this tissue seems to be especially troublesome.

The first step is a thorough defatting procedure, which we have found to be very important in order to obtain good yields and which seems to make the alkali treatment unnecessary. Probably it is the fatty material which prevents the extraction solution from coming in an intimate contact with the material in many cases. An outstanding example in this respect is the pleural tissue from which the heparin has been reported not to be extractable (Wegelius, 1956). However, we have found it possible to extract the heparin from this tissue after an extensive defatting procedure. Generally, we dry the frozen minced tissue at first in acetone and then defat it in a Soxhlet apparatus with acetone-ether (1:5). After this procedure, the tissue is minced again.

The next step is to extract the heparin with potassium thiocyanate (Snellman, Jensen and Sylvén, 1948). One point may be stressed here. In general tissue homogenates will very rapidly become acid and therefore the solutions must be buffered and the pH controlled. At slightly acid pH the heparin associates with proteins in the mixture, and that will cause trouble and even losses of substance. If the precaution is made to hold the pH at about 7.5 the whole time the proteins can easily be removed and, as far as we can judge, the heparin can be taken out and isolated quantitatively.

In the solution containing the heparin no other polyuronic acids will be found in the case of liver capsule. In other cases much of the chondroitin sulphuric acid will occur. The rest of the other polyu-

ronic acids remains in the residues which are extracted with a calcium chloride and then digested with ficin. By this treatment the hyaluronic acid and the remaining chondroitin sulphuric acid are released in about the same or slightly higher yields as in the alkaline treatments.

However, after these treatments residues are obtained. Alkali treatments of these residues at room temperature do not release any hexuronic acid but still there are some carbohydrates left undissolved. The method of Consden has been used on this material but no hexuronic acid could be detected, so it seems that no hexuronic acid-containing polysaccharide has been left as far as can be judged by this method.

Having thus obtained the different extracts containing the polysaccharides, the latter must be isolated and purified.

In the case of heparin, the solution has been precipitated with one volume of acetone and dissolved again in a small volume of buffer solution, and then reprecipitated with 60 per cent ethanol. By this procedure, much of the proteins are removed without any appreciable loss of heparin. The precipitate is then dissolved again in a suitable amount of phosphate buffer at pH 7.2 and a zone electrophoresis is performed.

We have found it most suitable to make the zone electrophoresis according to Flodin and Porath (1954) using an ethanolysed cellulose as supporting medium. This cellulose is easier to pack than Hyflo Super-Cel and does not adsorb the acid polysaccharides. With such a column even greater amounts of material can be separated in an easy manner and the column has a good separation power.

In the case of heparin, all substances of low electric charge, such as glycogen and proteins, are in a slow-moving zone. All hexuronic acid is found in one fast-moving fraction where the heparin and some nucleotide material are situated when skin or liver capsule are investigated. By an ethanol precipitation they were separated and heparin free of amino acids and nucleotide material was obtained. In one experiment 81 mg. of heparin, as determined by activity measurements, was found in the sample and 80 mg., determined in the same manner, was found in the final product, when liver capsule was investigated.

Also chondroitin sulphate and hyaluronate could be purified in the same manner here by dialysing and precipitating with ethanol before the zone electrophoresis.

From the discussion given we may conclude that in most cases it is unnecessary to begin with alkaline solutions. Such treatments do not seem superior to the other milder methods as a means of extraction for the acid mucopolysaccharides. In our experiments we have tried to investigate all solutions with the carbazole test of Dische (1947) in a qualitative manner to be sure that no hexuronic acid had been lost in the purification procedures. It thus seems that the hexuronic acids can be extracted and isolated in an as near as possible quantitative way and in a state which seems to be undegraded. If any further separation in components of the different substances or any other impurities than amino acids can occur has as yet not been investigated.

The carbohydrates in the residues have not as yet been further investigated. They must be very closely connected to proteins difficult to remove. It is possible that harder means must be used to isolate them. These residue carbohydrates may, from other points of view, have a great interest but they do not seem to contain any hexuronic acids.

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GROUP DISCUSSION

Dr. D. S. Jackson mentioned the use of quaternary ammonium salts for the specific precipitation of acid polysaccharides (Dr. G. E. Scott, personal communication). Complete separation of heparin, CSA and hyaluronic acid from each and from neutral polysaccharides can be achieved simply and rapidly.

Dr. Meyer pointed out that the Carbazole method might give erroneous results for uronic acid content. For example, iduronic acid and manuronic acid give much less coloration than glucuronic acid.

In reply to Dr. Consden, Dr. Snellman said that the Carbazole method was the only one he had used for determination of uronic acid.

COMBINATIONS *IN VITRO* COLLAGÈNE-MUCOPOLYSACCHARIDES ET MODIFICATIONS APPORTÉES À CES COMBINAISONS PAR DES SELS ET DES POLYOSIDES BACTÉRIENS[1]

Albert Delaunay et Suzanne Bazin

INTRODUCTION

On sait depuis plusieurs années que l'addition de sels ou de mucopolysaccharides à une solution de collagène donne souvent lieu à une précipitation. Tout récemment (Delaunay, Bazin et Hénon, 1956; Bazin, Delaunay et Hénon, 1956), nous avons montré que l'addition au collagène A de divers polyosides bactériens entraîne pareillement la formation d'un précipité.

Dans le travail ici présenté, nous rapporterons ce que nous avons constaté en étudiant les principaux caractères des combinaisons collagène-mucopolysaccharides et collagène-polyosides bactériens. Nous dirons aussi, sur la foi de nos observations, comment une combinaison normale collagène-mucopolysaccharide peut être modifiée quand elle doit prendre place en présence de sels ou de polyosides bactériens.

PARTIE EXPERIMENTALE

A. – *Matériel utilisé*

1. Collagène. Nous avons utilisé, pour toutes nos expériences, un collagène acido-soluble: le collagène A (Nageotte, 1927). Nos solutions étaient, toutes, limpides et âgées, au maximum, de 4 jours (Quantité de collagène dissous d'après les dosages d'azote et d'hydroxyproline: environ 0,8 mg. par ml.; pH de la solution standard: 4,3 à 4,5).

2. Mucopolysaccharides (M.P.). (*a*) Héparine. Ce produit purifié, provenait de la maison Hoffmann-Laroche. (*b*) Acide chondroïtine sulfurique. L'échantillon dont nous nous sommes servi nous avait été obligeamment fourni par le Pr. Fauré-Frémiet. Il avait été extrait du cartilage de veau selon les techniques de P. A. Levène. Avant usage, nous avons tenté de le purifier encore par 3 prépicipations successives (au moyen de 3 volumes d'alcool).

[1] Collagen-mucopolysaccharide combinations *in vitro* and how they are affected by bacterial polysaccharides and salts.

3. Polyosides bactériens. (*a*) et (*b*) Lipopolysaccharide (endotoxine) et polyoside typhiques. Ils ont été isolés, puis purifiés, selon les techniques classiques de A. Boivin (Boivin et Mesrobeanu, 1938). (*c*) Polyoside staphylococcique. Des staphylocoques qui ont poussé sur gélose sont, après lavages à l'eau physiologique, traités pendant 48 heures à $+4^{\circ}$ par de l'acide trichloracétique N/2. Après centrifugation, le liquide surnageant est dialysé puis concentré 10 fois. Cette préparation est soumise à des précipitations fractionnées par l'alcool, enfin desséchée sous vide. (*d*) Polyoside Cx. Pour obtenir celui-ci, nous avons suivi une méthode calquée sur celle de Anderson et McCarty (1951). Des pneumocoques rough du type II sont détruits rapidement par le désoxycholate de sodium. Les protéines microbiennes sont coagulées par la chaleur en milieu acétique. La fraction polyosidique est précipitée par l'alcool puis séparée des acides nucléiques au moyen de précipitations fractionnées par l'alcool en présence de Cl_2Ca.

4. Sels: chlorure et sulfate de sodium. Ces sels se trouvaient en solution dans l'eau distillée.

B. – *Technique opératoire*

1. A divers échantillons de notre collagène A a été ajoutée, sou volume égal, soit une solution de M.P., soit une solution de polyosides bactériens, soit une solution saline, soit, enfin, une solution contenant en mélange un M.P. et un sel ou un M.P. et un polyoside bactérien.

Les sels quand ils ont été utilisés isolément, l'ont été à différentes concentrations: pour le ClNa, de 0,85 à 0,0213 M, pour le SO_4Na_2, de 0,427 à 0,00427 M. En ce cas, nous avons laissé aux solutions leur pH naturel. Quand, avant l'expérience, les sels avaient été mélangés à un M.P., le pH du mélange était toujours ajusté à 4,3.

M.P. ou polyosides bactériens, employés isolément, ont été, de leur côté, ajoutés au collagène à diverses concentrations et à différents pH. Quand ils ont été ajoutés au collagène après mélange, un seul pH a été retenu: 4,3. Dans les mélanges M.P. – polyosides bactériens, les polyosides se trouvaient à doses variables, les M.P. à une seule dose qui était la dose précipitante optimum. Nous avons pris l'habitude de désigner sous ce nom la dose de M.P. (ou de polyosides) qui, ajoutée à une quantité donnée de collagène A, conduit à la formation d'un précipité qui contient tout le collagène présent et la majeure partie, sinon la totalité, du produit ajouté. L'absence de

collagène dans le liquide surnageant est démontrée par la négativité des réactions caractéristiques de l'hydroxyproline, l'absence des polyosides par la négativité de la réaction de Molisch après hydrolyse. Ajoutons que, dans ce cas, le liquide, surnageant, ne précipite plus en présence d'une nouvelle addition de sel (par exemple ClNa utilisé à doses normalement précipitantes) ou de polyosides.

2. Après addition au collagène A des diverses solutions définies ci-dessus, les tubes sont légèrement agités puis conservés pendant 3 heures à 18° C. A ce moment, leur contenu est examiné: on note s'il y a eu, ou non, précipitation. En cas de résultat positif, l'importance du précipité est estimée et son aspect macroscopique étudié.

3. Ceci fait, les précipités formés sont recueillis par centrifugation puis repris par de l'eau distillée amenée à différents pH. Nous avons déterminé de la sorte les marges de pH en dehors desquelles il y a dissolution des précipités.

Certains précipités, remis après lavage dans de l'eau amenée au pH du milieu où avait eu lieu la précipitation, ont été soumis à l'action du chauffage et du Cl_2Ca. Le chauffage, d'une durée de 3 minutes, a eu lieu à des températures variables. Nous avons noté la température la plus basse qui est suivie d'une contraction ou d'une dissolution du précipité. En ce qui concerne le chlorure de calcium, nous avons déterminé, dans chaque cas, la concentration limite qui est capable, à diverses températures, de dissoudre complètement le précipité examiné.

RESULTATS

A. – *Après addition au collagène d'héparine et d'acide chondroïtine-sulfurique* (*A.Ch.S*)

1. Principaux caractères des précipitations observées. Le lecteur trouvera, reporté dans le tableau I, l'essentiel de nos résultats.

TABLEAU I

CARACTÈRES DE LA PRÉCIPITATION OBTENUE APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, D'HÉPARINE OU D'ACIDE CHONDROÏTINE-SULFURIQUE

Nature de la substance utilisée pour la précipitation du collagène	*Limite d'activité de la substance précipitante*	*Dose précipitante optimum*	*Marges de pH entre lesquelles peut se produire une précipitation*	*pH du milieu où la précipitation paraît être optimum*
Héparine	0,02 mg.	0,10 mg.	1,5-8	4,3
Acide chondroï-tine sulfurique	0,05 mg.	0,20 mg.	2-8	4,3

Aux renseignements donnés par le tableau I, ajoutons les deux suivants. Une dose d'héparine supérieure à 1 mg/ml n'a pas précipité, dans nos conditions d'expérience, le collagène. Une dose d'A.Ch.S supérieure à 5 mg/ml n'a donné, dans les mêmes conditions qu'un faible précipité.

2. Principaux caractères des précipités obtenus. Prière de se reporter, pour les connaître, au tableau II.

TABLEAU II

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, D'HÉPARINE OU D'ACIDE CHONDROÏTINE-SULFURIQUE

Nature de la substance utilisée pour la précipitation du collagène*	*Aspect du précipité obtenu*	*Température de contraction*	*Marges de pH en dehors desquelles le précipité se dissout*	*Concentration limite (M) de Cl_2Ca capable de dissoudre complètement le précipité à 18° C.*
Héparine	Fibres longues et agrégées	48° C.	1,2-11	0,60
Acide chondroïtine-sulfurique	Fibres longues et agrégées	48° C.	1,5-11	0,06

* A dose précipitante optimum.

Ajoutons une précision. Des concentrations de Cl_2Ca, inférieures de 2 à 3 fois aux concentrations limites indiquées ci-dessus (qui sont valables pour des expériences faites à 18° C.), sont capables d'abaisser la température de contraction des précipités de 6 à 3° C.

B. – *Après addition au collagène de divers polyosides bactériens*

1. Principaux caractères des précipitations observées. Ils sont indiqués dans le tableau III.

TABLEAU III

CARACTÈRES DE LA PRÉCIPITATION OBTENUE APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE DE DIVERS POLYOSIDES BACTÉRIENS

Nature de la substance utilisée pour la précipitation du collagène	*Limite d'activité de la substance précipitante*	*Dose précipitante optimum*	*Marges de pH entre lesquelles peut se produire une précipitation*	*pH du milieu où la précipitation paraît être optimum*
Lipopolysaccharide (endotoxine) typhique	0,25 mg.	3 mg.	1,5-10,5	4,3
Polyoside typhique	0,50 mg.	1 mg.	5 -10	7,2
Polyoside staphylococcique	0,10 mg.	0,50 mg.	3 -7,2	4,3
Polyoside pneumococcique (Cx)	0,20 mg.	0,50 mg.	2,5-7,7	4,3

2. Principaux caractères des précipités obtenus. On les trouvera, exposés, dans le tableau IV.

TABLEAU IV

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, DE DIVERS POLYOSIDES BACTÉRIENS

Nature de la substance utilisée pour la précipitation du collagène*	*Aspect du précipité obtenu*	*Température de dissolution*	*Marges de pH en dehors desquelles le précipité se dissout*	*Concentration limite (M) de Cl_2Ca capable de dissoudre complètement le précipité a 18° C.*
Lipopolysaccharide (endotoxine) typhique	Fibres très courtes et non agrégées	56° C.	1-12	0,01
Polyoside typhique	Fibres mal formées. Précipité gélatineux	52° C.	5-12	0,01
Polyoside staphylococcique	Fibres longues et agrégées	54° C.	2-11	0,05
Polyoside pneumococcique (Cx)	Fibres courtes et non agrégées	54° C.	2,2-10,5	0,20

* A dose précipitante optimum.

Remarque: Des concentrations de Cl_2Ca inférieures de 2 à 3 fois aux concentrations limites indiquées ci-dessus (qui sont valables pour des expériences faites à 18° C.) sont capables d'abaisser la température de dissolution des précipités de 10 à 3° C.

C. – *Après addition au collagène de sels: ClNa et SO_4Na_2*

Le lecteur trouvera, indiquées dans le tableau V, à côté de la nature du sel utilisé, les concentrations moléculaires qui sont, ou non, précipitantes (+++ indique la formation d'un précipité abondant). Il connaîtra aussi le pH du mélange sel+collagène (le sel étant utilisé à la dose précipitante limite).

TABLEAU V

Nature du sel	*pH du mélange sel + collagène*	*Concentration moléculaire en sel*				
		0,00427	0,0427	0,085	0,213	0,427
ClNa	4,65	0	0	0	0	+++
SO_4Na_2	5,2	0	0	0	+++	+++

On voit, d'après le tableau V, que le collagène A n'est pas précipité quand les sels sont ajoutés à des concentrations inférieures à 0,427 M (ClNa) ou 0,213 M (SO_4Na_2) Ajoutons que les précipités isolés de la solution saline et placés dans l'eau distillée s'y dissolvent (cela, quel que soit le pH du milieu).

D. – *Après addition au collagène d'un mélange de M.P. (Héparine ou A.Ch.S.)+sels (ClNa ou SO_4Na_2)*

Rappelons que, dans ces mélanges, les M.P. se trouvaient à dose précipitante optimum (soit, pour l'héparine, 0,10 mg. et pour l'A.Ch.S., 0,20 mg.) et les sels à doses normalement précipitantes ou non.

1. Héparine+doses non précipitantes de ClNa: Précipitation (type héparine) normale en présence de 0,0213 M, faible en présence de 0,0427 M; pas de précipitation en présence de concentrations salines comprises entre 0,085 M et 0,213 M.

2. Héparine+dose précipitante de ClNa (0,427 M); pas de précipitation.

3. Héparine+doses non précipitantes de SO_4Na_2: Précipitation (type héparine) normale en présence de 0,0085 M, faible en présence de 0,0213 M; pas de précipitation en présence de 0,085 M.

4. Héparine + doses précipitantes de SO_4Na_2: Pas de précipitation en présence de 0,213 M; précipitation (type sel) peu abondante en présence de 0,427 M.

5. A.Ch.S. + doses non précipitantes de ClNa: Précipitation (type A.Ch.S.) normale en présence de 0,085 M. Obtention d'un précipité abondant mais de caractères particuliers en présence de 0,213 M. Ce précipité est particulier parce qu'il ne se contracte pas à 48° C. comme un précipité collagène-A.Ch.S. normal et qu'il ne se dissout pas dans l'eau distillée comme un précipité collagène-ClNa normal; il se dissout à 56° C.

6. A.Ch.S. + dose précipitante de ClNa (0,427 M): Précipitation (type sel) normale.

7. A.Ch.S. + doses non précipitantes de SO_4Na_2: Précipitation (type A.Ch.S.) normale en présence de 0,0427 M et de concentrations salines plus faibles. Obtention d'un précipité abondant mais de caractères particuliers en présence de 0,085 M. Ce précipité, particulier parce qu'il ne se contracte pas à 48° C. et qu'il ne se dissout pas dans l'eau distillée, se dissout à 52° C.

8. A.Ch.S. + doses précipitantes de SO_4Na_2: Obtention d'un précipité peu abondant et de caractères particuliers (particuliers pour les mêmes raisons que celles données ci-dessus: cf. paragraphes 5 et 7) en présence de 0,213 M. En présence de 0,427 M, précipitation (type sel) peu abondante.

E. – *Après addition au collagène d'un mélange M.P. (héparine ou A.Ch.S.) + polyosides bactériens*

Le lecteur trouvera dans le tableau VI l'essentiel des résultats que nous avons recueillis quand, dans le mélange, mucopolysaccharide et polyoside bactérien étaient utilisés, tous deux, à la dose précipitante optimum. Il trouvera aussi, dans les lignes supérieures de ce tableau, le rappel des faits que l'on constate quand on utilise isolément, à cette même dose, mucopolysaccharides et polyosides; une étude comparative sera de la sorte grandement facilitée.

Voici par ailleurs, les résultats que nous avons obtenus en ajoutant au collagène A des mélanges mucopolysaccharides + polyosides bactériens; les premiers étant toujours à dose précipitante optimum, les seconds au contraire étant tantôt à dose forte (c'est-à-dire supérieure), tantôt à dose faible (c'est-à-dire inférieure à la dose précipitante optimum).

1. Héparine + lipopolysaccharide typhique. (*a*) à dose forte

TABLEAU VI

CARACTÈRES DES PRÉCIPITÉS OBTENUS APRÈS ADDITION, À UNE SOLUTION DE COLLAGÈNE A, DE MUCOPOLYSACCHARIDES, DE POLYOSIDES BACTÉRIENS OU D'UN MÉLANGE MUCOPOLYSACCHARIDE + POLYOSIDE BACTÉRIEN (TOUS CES CORPS ÉTANT UTILISÉS À LA DOSE PRÉCIPITANTE OPTIMUM)

Nature des substances précipitantes utilisées	*Importance du précipité obtenu*	*Aspect du précipité obtenu*	*Action de la chaleur sur le précipité*	
			Température de contraction	*Température de dissolution*
Héparine	+++	Fibres longues, agrégées	48° C.	
Acide chondroïtine-sulfurique	+++	Fibres longues, agrégées	48° C.	
Lipopolysaccharide (endotoxine) typhique	+++	Fibres très courtes, non agrégées		56° C.
Polyoside typhique*	+++	Fibres mal formées (précipité gélatineux)		52° C.
Polyoside staphylococcique	+++	Fibres longues, agrégées		54° C.
Polyoside pneumococcique (Cx)	+++	Fibres courtes, non agrégées		54° C.
Héparine + lipopolysaccharide (endotoxine) typhique	++±	Fibres courtes, non agrégées		56° C.
Héparine + polyoside typhique	+±	Fibres longues (précipité non gélatineux)	50° C.	
Héparine + polyoside staphylococcique	0			
Héparine + Cx	+++	Fibres, longues agrégées	48° C.	
Acide chondroïtine-sulfurique + lipopolysaccharide (endotoxine) typhique	++±	Fibres courtes, non agrégées		56° C.
Acide chondroïtine-sulfurique + polyoside typhique	++	Fibres longues (précipité non gélatineux)	50° C.	
Acide chondroïtine-sulfurique + polyoside staphylococcique	+++	Fibres longues, agrégées	48° C.	
Acide chondroïtine-sulfurique + Cx	+++	Fibres longues, agrégées	48° C.	

* Observations faites exceptionnellement, non à pH 4,3, mais à pH 7.

(5 mg./ml.): obtention d'un précipité (type endotoxine) normal. (*b*) à dose faible (1 mg./ml.): obtention d'un précipité particulier qui diffère notamment du précipité collagène-héparine habituel par l'aspect des fibres (elles sont moins longues) et sa température de contraction (50° C. au lieu de 48° C.). Ajoutons que la contraction se fait, non en une seule masse, mais en petits grains.

2. Héparine + polyoside typhique. (*a*) à dose forte (2 mg./ml.): précipitation anormalement faible; le précipité se contracte à 50° C. (*b*) à dose faible (0,25 mg./ml.): précipitation (type héparine) normale.

3. Héparine+polyoside staphylococcique. (*a*) à dose forte (2 mg./ml.) et (*b*) à dose faible (0,20 mg./ml.): pas de précipitation.

4. Héparine+polyoside pneumococcique Cx. (*a*) à dose forte (1 mg./ml.): obtention d'un précipité type héparine mais qui se contracte à 50° C. (*b*) à dose faible (0,2 mg./ml.): obtention d'un précipité (type héparine) normal.

5. A.Ch.S.+lipopolysaccharide typhique. (*a*) à dose forte (5 mg./ml.): obtention d'un précipité (type endotoxine) normal. (*b*) à dose faible (1 mg./ml.): obtention d'un précipité (type A.Ch.S.) normal.

6. A.Ch.S.+polyoside typhique. (*a*) à dose forte (2 mg./ml.): obtention d'un précipité type A.Ch.S. mais qui se contracte à 50° C. (*b*) à dose faible (0,25 mg./ml.): obtention d'un précipité (type A.Ch.S.) normal.

7. A.Ch.S.+polyoside staphylococcique. (*a*) à dose forte (2 mg./ml.) et (*b*) à dose faible (0,20 mg./ml.): obtention d'un précipité (type A.Ch.S.) normal.

8. A.Ch.S.+polyoside pneumococcique Cx. (*a*) à dose forte (1 mg./ml.): obtention d'un précipité type A.Ch.S. mais qui se contracte à 50° C. (*b*) à dose faible (0,2 mg./ml.); obtention d'un précipité (type A.Ch.S.) normal.

DISCUSSION

1. On savait déjà que des mucopolysaccharides comme l'héparine et l'A.Ch.S sont capables de précipiter tous deux un collagène soluble en donnant naissance à de belles fibrilles. A la lumière de nos observations, on peut ajouter maintenant les précisions suivantes:

– Les marges de pH entre lesquelles peut se produire une précipitation de cet ordre sont larges (de 1,5 ou 2 à 8); pH optimum: 4,3.

– Les précipités obtenus contiennent à la fois le collagène et le M.P. ajouté (ceci étant démontré par le double épuisement du liquide surnageant).

– Dans les précipités, il y a non seulement présence mais encore combinaison des deux principes chimiques. Comme preuve, nous donnerons les résultats de l'expérience suivante. A des échantillons d'une même solution de collagène A sont ajoutées des doses variables d'héparine (0,05, 0,10, 0,25 et 0,50 mg. par ml. de collagène). Les précipités recueillis sont lavés et desséchés sous vide. Résultats du dosage de l'hexosamine dans 1 mg. de précipité sec: 26 μg 7, 27 μg 1;

27 µg 8, 27 µg 3. Quatre chiffres aussi voisins n'auraient pu être trouvés si une véritable combinaison chimique n'était pas en cause (dans le collagène A précipité par le ClNa, pas d'hexosamine décelable par la méthode que nous avons utilisée).

– Soumis à l'action de la chaleur, les précipités collagène-héparine ou collagène A.Ch.S. se comportent de la même façon puisque tous deux se contractent à 48° C. En revanche, le premier est plus aisément dissociable par le Cl_2Ca; il serait donc moins stable.

2. Des polyosides bactériens comme le lipopolysaccharide typhique, les polyosides typhique et staphylococcique, enfin le polyoside pneumococcique Cx peuvent, au même titre que les M.P. mentionnés plus haut, précipiter *in vitro* le collagène A. Cette fois encore, à une exception près, nous avons insisté sur cette exception qui concerne le polyoside typhique dans un travail précédent (Bazin, Delaunay, Hénon, 1956), on peut préciser que les précipitations traduisent de véritables combinaisons chimiques. Combinaisons, toutefois, très particulières puisque, non seulement elles diffèrent totalement des combinaisons collagène-M.P., mais qu'elles diffèrent encore profondément entre elles. Ainsi:

– La dose précipitante optimum varie nettement d'un polyoside bactérien à l'autre; en tout cas, cependant, nous l'avons toujours trouvée supérieure, et souvent très supérieure, aux doses correspondantes pour l'héparine et l'A.Ch.S.

– Les marges de pH qui permettent une précipitation sont, de leur côté, non moins variables.

– Variabilité encore, en ce qui concerne l'aspect et les propriétés des précipités obtenus. On observe la formation, tantôt de belles fibrilles, tantôt d'un simple dépôt gélatineux. Mais quels qu'ils soient, les précipités recueillis, quand ils sont chauffés, se dissolvent. Par ailleurs, ils sont aisément dissociables par le Cl_2Ca.

On peut dire, dans ces conditions, que les combinaisons collagène-polyosides bactériens sont moins solides que les combinaisons collagène-M.P.

3. Autre fait nouveau apporté par notre travail: une combinaison normale collagène-M.P. (héparine ou A.Ch.S.) peut être inhibée par la présence dans le milieu de sels (ClNa ou SO_4Na_2), ceux-ci étant utilisés à doses précipitantes et même à doses non précipitantes. Cette action inhibitrice est particulièrement nette dans le cas de l'héparine.

Pareille observation mérite sans doute d'être rapprochée de

certaines, autres recueillies dans des domaines voisins. Ainsi, Blumberg, Oster et Meyer (1955) ont signalé que, mis en présence de faibles concentrations de ClNa, l'acide hyaluronique donne des agrégats particulaires, et que ces agrégats, au contraire, se dissocient quand le ClNa présent dans le milieu atteint une concentration physiologique. Des remarques du même ordre ont été faites, avec l'acide chondroïtine sulfurique par Mathews (1953). L. B. Jaques (1943) a remarqué pour sa part que le chlorure de sodium, utilisé à une concentration de 5%, inhibe fortement la combinaison héparine protamine (en présence de ClNa, 5 fois moins d'héparine se trouvent fixés). D'après S. S. Cohen (1942), la précipitation d'une nucléoprotéine (il s'agissait en l'occurrence du virus de la «tomato bushy stunt») par l'héparine, l'A.Ch.S. et l'acide hyaluronique, est inhibée par 0,1 M de ClNa; elle reprend place, cependant, en présence d'une plus grande quantité de sel. Ajoutons enfin que, selon J. Badin et M. Schubert (1955), la précipitation qui se forme quand on met en présence de l'acide chondroïtine-sulfurique et des globulines sériques voit son intensité diminuée de 50% par 0,06 M de ClNa; elle serait inhibée totalement par 0,2 M du même corps.

4. Les combinaisons collagène-M.P. (héparine ou A.Ch.S.) cessent aussi de se faire normalement *in vitro* quand, dans le milieu, se trouve un polyoside bactérien comme le lipopolysaccharide typhique, les polyosides typhique et staphylococcique, enfin le polyoside pneumococcique Cx.

Les combinaisons collagène-héparine sont surtout troublées quand, dans le milieu, se trouve un lipopolysaccharide typhique ou un polyoside staphylococcique. Parfois, on ne note aucune précipitation. Si elle se produit, elle est le plus souvent peu abondante; encore le précipité formé est-il en général du type collagène-polyoside bactérien (ex: collagène-lipopolysaccharide typhique).

Les combinaisons collagène-A.Ch.S. sont, elles aussi, troublées surtout par la présence du lipopolysaccharide typhique et du polyoside staphylococcique. Dans l'ensemble, toutefois, elles sont troublées moins nettement que les précédentes, ce qui étonne d'ailleurs un peu, attendu que, d'après l'action du Cl_2Ca, on serait tenté de les croire normalement moins stables.

5. Motifs d'intérêt des observations mentionnées ci-dessus. Ils sont, à notre avis, divers. Nous les résumerons ainsi.

(*a*) D'assez nombreux auteurs pensent à l'heure actuelle que des mucopolysaccharides sont un des éléments du ciment qui unit, dans

la fibre conjonctive, les fibrilles et les filaments. Nous ne pouvons pas dire que nos recherches personnelles apportent un argument supplémentaire en faveur de cette conception. Nous croyons pourtant qu'en mettant en évidence la production *in vitro* de véritables combinaisons chimiques entre collagène et M.P., elles offrent le grand avantage de souligner l'affinité extraordinaire que possèdent ces substances les unes pour les autres. Que celles-ci se « lient » dans l'organisme comme on les voit se « lier » dans un milieu artificiel, on distingue mal, en vérité, ce qui pourrait empêcher de l'admettre.

(*b*) Dans un rapport récemment présenté (Delaunay, Bazin, Hénon et Fauve, 1956), nous avons émis l'hypothèse que certaines maladies dites « du collagène » pourraient avoir précisément pour cause des liaisons devenues défectueuses entre la protéine collagène et les molécules de M.P. Mais pourquoi ces liaisons seraient-elles défectueuses ou, si l'on veut dangereuses? Les faits expérimentaux que nous avons relatés ci-dessus à propos des sels et des polyosides bactériens apportent une réponse au moins plausible. Ces liaisons deviendraient défectueuses parce que se trouveraient à un moment donné, dans la trame conjonctive, des éléments qui sont capables, eux aussi de contracter des liaisons avec le collagène ou les M.P. Se ferait jour ainsi une action compétitive entre plusieurs substances, d'où, en dernière analyse, formation d'un ciment mal lié. Au nombre de ces substances, nous n'hésitons pas, en nous fondant sur nos résultats *in vitro*, à ranger des sels (ils seraient présents, localement, à des concentrations trop faibles ou trop fortes) et des principes bactériens divers (par exemple, polyosides).

(*c*) Il est toujours dangereux d'extrapoler et de vouloir imaginer ce qui peut se passer *in vivo* d'après ce qu'on voit *in vitro*. Nous le savons mieux que quiconque. Il n'en reste pas moins vrai qu'au point atteint par nos recherches, nous faisons à l'hypothèse mentionnée le plus large crédit.

RESUMÉ ET CONCLUSION

Les auteurs ont étudié les combinaisons qui se forment *in vitro* entre le collagène A d'une part, des mucopolysaccharides (héparine, acide chondroïtine-sulfurique) ou des polyosides bactériens (lipopolysaccharide typhique, polyosides typhique et staphylococcique, polyoside pneumococcique Cx) de l'autre.

Ils ont ensuite montré comment des combinaisons collagène-

mucopolysaccharides peuvent être troublées par la présence dans le milieu de sels (ClNa, SO_4Na_2) ou de polyosides bactériens.

En conclusion, ils ont émis une hypothèse d'après laquelle certaines « maladies du collagène » pourraient résulter simplement de liaisons devenues défectueuses entre la protéine collagène et les molécules de M.P., ces liaisons défectueuses tenant à l'entrée en jeu intempestive (action compétitive) de sels ou de principes bactériens.

SUMMARY

1. When to a solution of collagen A a solution of heparin or chondroitin sulphuric acid is added, a precipitate forms. This fact has been known for several years, by electron microscopists in particular, but has never been the object of a systematic study.

We have determined, for the two mucopolysaccharides quoted, the minimum precipitating dose, the optimum precipitating dose, the limits of pH between which precipitation occurs, the pH of the medium for apparent optimum precipitation, etc. We have also studied systematically the principal characters of the precipitates obtained (appearance, temperature of contraction, etc.). We were also able to show that the precipitates are the result of a true chemical combination between the collagen and the precipitating substance.

2. A precipitate may also be obtained when bacterial polysaccharides are added to a solution of collagen A. We have discovered this fact by using typhoid lipopolysaccharide (endotoxin), typhic and staphylococcal polysaccharides, or the Cx polysaccharide extracted from pneumococcus (rough). The precipitates obtained in this way differ not only from those given by chondroitin sulphuric acid and by heparin but also they differ from each other. For each bacterial polysaccharide we have defined the conditions for precipitation and the nature of the precipitates formed.

3. Having established these facts, we have studied what happened when heparin or chondroitin sulphuric acid was added to collagen A in the presence of salts or of a bacterial polysaccharide. The results are as follows:

(*a*) In the presence of salts (NaCl, sodium sulphate) we obtained either a very faint precipitate having different properties or no precipitate at all. The variability of the results depended on the nature of the mucopolysaccharide used and on the nature and amount of the salts present in the medium.

(*b*) The combinations collagen-heparin or collagen-chondroitin sulfuric acid are also altered when they take place in the presence of a bacterial polysaccharide. Precipitation may be inhibited. More often precipitation occurs but is of an unknown type.

4. The authors have attempted to determine the significance of these observations both in biochemistry and pathology.

GROUP DISCUSSION

DR. MEYER asked whether the base binding power of the solutions of polysaccharides and the acid binding power of the collagen solutions had been determined. He wondered whether the polysaccharide did in fact combine stoichiometrically with collagen A.

DR. GROSS pointed out that a variety of substances, polysaccharides and non-polysaccharides, precipitate with collagen, sometimes giving structured or non-structured fibrils and sometimes short 'crystallites'.

DR. DELAUNAY agreed that collagen is precipitated by very many substances. He felt that there was, however, a chemical combination between collagen and mucopolysaccharides.

DR. D. S. JACKSON referred to experiments by Dr. Bungenberg de Jong working at Utrecht. This worker, up to the present date, he said, had studied the interaction of a large number of polyelectrolytes and neutral polymers with every possible combination of anions and cations and had evoked a theoretical explanation for these phenomena. Dr. Delaunay's observations could be a specific example of the general phenomenon of colloid interaction.

DR. BALÓ remarked that it was assumed that collagen fibres originated from the cells whereas the experiments reported could be interpreted as showing that fibres could be formed without the intervention of cells.

DR. FITTON JACKSON pointed out that one should always take into account the state of the collagen in solution. It had been suggested that the cells synthesize the precursors, e.g. the amino acids which are built up into peptides which thence constitute the collagen molecule, and these are ultimately incorporated into the collagen fibrils. On the other hand, it is the polypeptide chains which are in solution and are precipitated by the mucopolysaccharides or by other methods.

DR. ROBB-SMITH said that the experiments of Dr. Delaunay and Mlle Bazin are 'type' experiments which present considerable points of interest. However, it was very difficult to translate them directly into terms of pathology or fibrillogenesis.

DR. GROSS agreed and pointed out that Dr. Delaunay had been very cautious in his interpretation. It was tempting to assume that because two

phenomena take place simultaneously in tissues they must be causally related. This is not necessarily the case, and it may well be that the mucopolysaccharides in the tissues have something to do with other functions of these tissues and have nothing to do with the formation of collagen fibrils. He felt that there was at present no proof that mucopolysaccharides are specifically involved in the formation of collagen fibrils. Indeed, *in vitro* it is possible to obtain fibrils, apparently identical to native fibrils, in the absence of mucopolysaccharides. One should be cautious in speaking of chemical combinations in the present case. It may be that long polymers interact mechanically or, at least, non-stoichiometrically.

DR. NEUBERGER agreed and said that when two charged substances are present in solution they will interact with one another by Coulomb and other forces and, depending on circumstances, the solubility of the mixture might be greater or less than that of either of the components. This could not be predicted at present.

DR. CONSDEN asked how it was possible to determine the combinations of acid-soluble collagen with polysaccharides at higher pH values, since acid-soluble collagen is precipitated above pH 7.

Dr. BAZIN answered that the existence of a combination between collagen (in solution at pH 4.3) and a polysaccharide (in solution at a pH higher than 5) is shown by the characteristic quality of the precipitate obtained.

In conclusion, DR. DELAUNAY agreed with all speakers and in particular with Dr. Gross and Dr. Neuberger. He had presented facts which are easily reproducible and submitted them to the meeting. As to the interpretation of these facts, it was another matter and would be the task of tomorrow.

MICRO-ANATOMY AND REACTIONS TO INJURY OF VASCULAR ELASTIC MEMBRANES AND ASSOCIATED POLYSACCHARIDES

THEODORE GILLMAN, MICHAEL HATHORN AND JACK PENN

During the last six years the investigations in this laboratory have been directed towards understanding the histogenesis and histochemistry of repair. Hitherto, analyses have been made primarily of the healing, in animals and in man, of cutaneous injuries, ranging from acute trauma to chronic damage due to ultraviolet and roentgen irradiations (Gillman *et al.*, 1953; 1955c; Gillman and Penn, 1956a, b). Apart from understanding the healing of cutaneous lesions and of some aspects of epithelial-connective tissue relations in the pathogenesis of skin carcinomas, it has always been anticipated that some light may be thrown on repair processes *generally*, irrespective of their location in the body. Thus, it is considered that an appreciation of the reactions to acute and chronic lesions in the skin may elucidate, in some measure, the basic processes involved in repair, as opposed to regeneration, by connective tissues, of lesions in the liver, arteries, myocardium, etc.

Careful study of the healing of various types of cutaneous injuries revealed that, following acute trauma (extending deep to the elastic fibrils of the stratum papillaris), scar formation was invariable (Gillman *et al.*, 1955c; Gillman and Penn, 1956a, b) and that, contrary to some expressed opinions (e.g. Converse and Robb-Smith, 1944), 'normal' elastic fibres did not reappear in the scar tissue for months or even years. Fibres, stained with accepted elastic stains, frequently appear in longstanding scars. But the morphology of these fibres, their arrangement in relation to the collagen bundles in scars and their other tinctorial reactions previously reported upon (Gillman *et al.*, 1954; 1955a, b) as well as some of the other characteristics reported on in the literature (Lansing, 1955) indicated that these were probably *not* identical with the 'normal' cutaneous elastic *fibrils*. On the basis of their morphology and tinctorial reactions these elastic-like fibres were shown to be distinguishable from 'normal' elastic fibres and were named *pseudo*-elastic fibres. Such pseudo-elastic fibres

(P.E.F.s) were shown to be the main components of senile elastosis and the so-called increase in elastic tissue or 'basophilic collagen' regularly encountered and commonly described in the skin, in association with chronic radiation dermatitis in man. The experimental production, in man *in vivo*, of localized patches of such pseudo-elastic fibre formation in the dermis has also been reported upon from this laboratory (Gillman *et al.*, 1953; 1955b).

In attempting to characterize them, these fibres were compared with the thick wavy elastic laminae or membranes in the aorta, carotid and other large arteries. Apart from revealing that morphologically and tinctorially identical P.E.F.s could also be found in the media of arteries undergoing various types of degeneration (Gillman *et al.*, 1955a) it appeared to us that the elastic membranes in healthy arteries also required further morphological study in order to elucidate their structure as well as to ascertain their reactions to injury.

The objects of the present report are to outline briefly firstly, some of the morphological and tinctorial reactions of vascular elastic membranes (V.E.M.s) in healthy arteries and secondly, the changes which these undergo following injury in man, and especially (in different parts of the arterial tree) in rats given toxic doses of calciferol. Some of the implications of these findings will also be reviewed.

We are painfully aware of the limitations of staining methods in defining chemical changes in tissues, but nevertheless we firmly believe that careful observations, such as those recorded here, often form the starting-point for investigations which will ultimately lead to clarification of fibre structure, by the use of the most modern histochemical, chemical and biophysical methods and criteria, most of which are not available to our group. Some indication of what may be achieved if attempts are made to explain the tinctorial reactions, used widely, effectively, and for many years by histologists and histopathologists, is available from a recent outstanding publication by Glegg, Eidinger and Leblond (1953). By the combined use of histological, chemical and physical methods, these investigators were able to provide a satisfactory chemical explanation for some of the differences in staining properties of reticular and collagenous fibres.

We sincerely hope that the precise nature of the tinctorially definable alterations in connective tissue fibres in dermis, blood

vessels and gall bladder, outlined previously and below, will also soon be elucidated in terms of the chemistry and ultrastructure of connective tissue fibres.

MATERIAL AND METHODS

Aortae, carotids, pulmonary and other medium and small arteries and arterioles were obtained, at post mortem, from human subjects immediately after acute traumatic death (10–30 minutes) or dying from various diseases. Serial wax sections of these vessels, fixed in formalin, were stained by the methods previously outlined (Gillman *et al.*, 1953; 1955a, b; Gillman and Penn, 1956a, b) to demonstrate cytology and elastic, collagen and reticulin fibres by the periodic-acid Schiff (P.A.S.) and von Kossa (V.K.) routines – to demonstrate polysaccharides and mineralization respectively – and by the alcoholic toluidine blue method recommended by Glick (1949) for identifying metachromatic mucopolysaccharides in connective tissue ground substance and in mast cells.

The experimental material comprised heart and coronary vessels, aorta (4 parts – i.e. ascending, thoracic, pre-renal abdominal and post-renal abdominal), spleen, stomach, intestine and other tissues from male and female rats, weighing 160 to 210 grammes, receiving 24,000–72,000 units of calciferol daily, by mouth, for five consecutive days only. Thereafter post mortem studies were conducted on moribund animals or rats sacrificed between the fourth and the forty-third day after the initiation of the experiments. Tissues from these animals were prepared in the same ways as outlined above for human materials.

OBSERVATIONS

1. *Morphology and Histochemistry of Normal Elastic Membranes*

Some of the staining reactions of vascular elastic membranes (V.E.M.s) have been recorded previously (Gillman *et al.*, 1955a). In particular it has been shown that these membranes seem to be composed of axial homogeneous 'cores' having tinctorial reactions different from the material which seems to constitute a thin, surrounding coating in larger vessels or an enmeshing spiral of fibrils in smaller vessels (see below). Only some of the staining qualities of these 'cores' are the same as those of the fine elastic fibrils encount-

ered in the dermal stratum papillaris and in the other loose areolar connective tissues.

Distinct differences in staining between the axial cores of the V.E.M.s and fine, elastic fibrils have been demonstrated and previously enumerated (Gillman *et al.*, 1955a, b). Moreover, in reticulin preparations of human arteries, a fine and almost continuous sheath of reticulin fibres is regularly encountered, extending along the outer borders of the wavy cores of the V.E.M.s (Fig. 1). From these outer reticulin sheaths, numerous fine reticulin fibrils extend into the surrounding interstitial collagen and unite with the perimuscular reticulin network. In Wilder preparations, counterstained with Van Gieson's stain, the 'core' of the V.E.M.s, lying within the reticulin 'sleeve', stains yellow, while the neighbouring collagen stains bright red. In the rat's aorta (Fig. 2) the reticulin sheath is finer and seems to be constituted of comma-shaped granules, the 'tails' of the commas extending into the intermembranous reticulin and collagen meshwork (see legend and lower portion of Fig. 19 at *a*).

Polysaccharides (as demonstrated with P.A.S.) form a distinct thin membrane-like coating around the axial cores, corresponding closely with the distribution of reticulin. Material staining metachromatically with toluidine blue (in the human aorta and carotid) is irregularly distributed in the intermembranous collagen, but is distinctly aggregated as a coating around the pale blue-staining V.E.M.s. In Rinehart-Abul Haj preparations (a modified Hale's ferrocyanide method) the deep blue-staining mucopolysaccharides are less widely distributed and regularly form a coating around the yellow-staining membranes. In the normal rat's aorta the perimembranous reticulin sleeve is finer and more granular than that in man (Fig. 2), the P.A.S. positive sleeve around the V.E.M.s is distinct although thin, but only minimal amounts of metachromatic material are detectable, scattered among the inter-membranous muscle bundles, and forming an irregular interrupted peri-membranous sheath.

Staining with Mallory's Phosphotungstic Acid Haematoxylin (Mallory's P.A.H.) demonstrates, in human aortic V.E.M.s, a positively staining membrane with occasional 'moth-eaten' clear spaces along its length. This blue-stained sleeve around the wavy membrane is closely hugged by the surrounding orange-staining collagen. In the human aorta this sleeve around the elastic mem-

branes (which stains deep blue to purple with Mallory's P.A.H.), is virtually unbroken. However, further insight into their possible structure is obtained from the study of smaller arteries in man and of the aorta in the rat. In the internal elastic membranes of the small visceral arterioles in man (Fig. 3) the material, staining blue with Mallory's P.A.H., appears granular; between these granules a core of material, staining orange (like collagen), can easily be seen, in good preparations. The blue-staining material seems to be constituted of thin fibrils wound spirally around the collagen-like core of the internal elastic membranes. This interpretation is supported by several facts. Firstly on focusing up and down, on such internal elastic membranes, the blue 'dots' seem to join one another, giving the optical impression of spiralling, an impression not transmissible in stills taken at one plane of focus. Secondly, in oblique sections of such membranes (Fig. 4) the clear homogeneous collagen-like orange-stained core is either flanked by blue fibrils (Fig. 4*a*) or has distinct blue stripes (Fig. 4*b*). This impression, gained from the study of medium-sized arteries, is reinforced by the findings in terminal arterioles. In the latter, the collagen-like core seems thicker and more homogeneous, while the surrounding spiral of blue-staining fibrils seems to be less tightly coiled. In sections, stained with elastic stains (orcein, Weigert's resorcin-fuchsin or Verhoeff's methods), the positively stained material is coarsely granular (Fig. 5), a non-staining structure being occasionally visible between these coarse granules. However, with Mallory's P.A.H. (Fig. 6 – a serial section of the same arteriole shown in Fig. 5), the collagen-like portion of the internal elastic membrane is prominent, being flanked by rows of very fine blue-staining granules.

Thus, it would seem, that in man, the collagen-like core of the elastic membranes, in larger arteries, is surrounded by an almost continuous, fairly thick coat of some other material (perhaps elastin-like or reticulin-like) while, as the arteries become smaller, so the collagen-like core becomes thicker and is surrounded by a thinner coating of this other material, which now forms a spiral of blue-staining fibrils – this spiral becoming progressively more loosely coiled and constituted of finer fibrils as one proceeds to the smallest branches of the arterial tree.

These findings and interpretations, concerning the arterial elastic membranes in man, are further confirmed from a study of the arteries in rats. In the normal aorta of the rat, the V.E.M.s are clearly

constituted of a core of material staining orange with Mallory's P.A.H. These cores are surrounded and connected with one another by fine blue-staining fibrils (Fig. 18) having a disposition different from reticulin (compare Figs. 2, 18 and 19). Following injury, the 'cores' become thicker and the inter- and peri-membranous blue-staining fibrils seem to disappear in a patchy fashion (compare the upper and lower parts of Fig. 18). In orcein-stained preparations of the normal rat's aorta, the cores of the V.E.M.s are tinted pale brown while the outer portions are heavily stained. These differences in staining are particularly clearly visible in oblique sections of these V.E.M.s, in which deep brown stripes can be seen spiralling around the paler staining cores.

Thus, from the histological evidence here outlined, it seems that the V.E.M.s in arteries: (1) Are not homogeneous in structure, being constituted of a core of collagen-like material, sleeved by polysaccharide and possibly some other fibrillar element similar to elastin. (2) Differ tinctorially, morphologically and histochemically from elastic fibrils found in skin and loose areolar connective tissue. (3) Are structurally closely inter-related with the surrounding reticulin, collagen and polysaccharides. (4) Structures delineated with elastic stains, and apparently homologous in the larger arteries of the rat and man, differ morphologically and tinctorially when other criteria are used.

These morphological and tinctorial findings in healthy arteries are substantiated by and permit a fuller understanding of the reactions to injury.

2. *Reactions of Elastic Membranes to Injury*

In man, damage of elastic membranes is invariably followed by reactions indistinguishable from those seen in healing cutaneous injuries, i.e. fibroblast, giant cell, mucopolysaccharide and collagen accumulation (Fig. 7) with subsequent scar formation. These are the reactions of repair of injury as opposed to regeneration. Reactions, similar to those here portrayed, have also been described recently in the carotid artery 'elastic granuloma' in man. The damaged elastic membranes seem to act as foreign bodies evoking giant cell formation (Fig. 8). Walker and Wirtschafter (1956) have also demonstrated, in recent months, that the widespread lysis of vascular elastic membranes, induced by feeding sweet-pea seed flour to rats, is invariably followed by extensive fibroblastic reactions with scar

formation. Unfortunately, the latter authors made no mention of the reactions of the elastic fibrils in skin, lungs, elastic cartilages and areolar connective tissues.

In man, damage of the media of smaller arteries, as previously described (Gillman *et al.*, 1955a), may be associated with the accumulation of typical P.E.F.s.

In rats, treated for only five consecutive days with toxic doses of calciferol, the following sequence of histologically and histochemically demonstrable changes are regularly encountered. Mineralization (von Kossa positive material) of the myocardium and of the intimal portions of the coronary arteries (Fig. 9) ensues within 4-6 days of the first dose of calciferol. These cardiac lesions usually resolve rapidly so that by the tenth day von Kossa positive material is no longer detectable either in the myocardium or in the coronary arteries. The former now show marked round cell and fibroblastic infiltrations (with or without giant cell formation), while the coronaries now present a markedly thickened homogeneous (hyaline) strongly P.A.S. positive intima (Fig. 10). Between the fifth and twelfth days the internal part of the media, first of the arch (Fig. 15), then of the thoracic and later of the abdominal aorta becomes heavily mineralized, as may also the alveolar portions of the lungs and usually, in addition, parts of the gastric mucosa, the musculature of the stomach and of the colon. The mineralization of the alimentary tract is usually most marked between the eleventh and eighteenth days of the experiment. After this time, most (but not all) of the von Kossa positive material disappears from the various parts of the aorta, and from the alimentary tract, so that, by the fortieth day (i.e. 35 days after the last dose of calciferol) only isolated sections of the ascending and thoracic aorta retain von Kossa positive material (Fig. 20). The cardiac muscle regenerates completely, while the coronary arteries and the severely damaged portions of the aorta undergo varying degrees of fibrosis.

Details of these reactions, which are similar to those described by other authors (Sohl *et al.*, 1930; Ham and Portuondo, 1933; Ham and Lewis, 1934) have been presented elsewhere (Gillman *et al.*, 1956). Pertinent to the present discussion are the changes in the coronary arteries, aorta and smaller vessels – the latter not having been mineralized at any stage of the experiments.

At the outset it must be stated that in *all* loci, accumulations of von Kossa positive material are consistently associated with marked

increases in P.A.S. positive material (compare Figs. 9 and 10). Resorption of the von Kossa positive material (apart from that in the myocardium) is followed by the accumulation of pools of alcohol-resistant metachromatic substance – presumably mucopolysaccharides (M.P.S.) with or without fibrosis (Figs. 20 and 21) – depending on the intensity and duration of the damage.

On superficial examination, the von Kossa positive material seems to be deposited within the elastic membranes. However, careful study of the reactions preceding, during and succeeding mineralization reveals that the calcium (and other von Kossa positive mineral salts) is not deposited within the elastic membranes, but rather around them. This is clearly demonstrated for the aortic elastic membranes in Figs. 16 and 17. Similarly, absorption of these minerals, coating the vascular elastic membranes (V.E.M.s), is followed by a marked accumulation of mucopolysaccharides around and between the previously mineralized V.E.M.s (Figs. 20 and 21). Apparently, only if the mineralization is very severe or prolonged (as occurs especially in the first part of the aorta of rats, fed larger doses of calciferol) do the V.E.M.s themselves become disrupted, leading to irregularities in their arrangement in the vascular walls, very marked mucopolysaccharide accumulations, frequently associated with ulceration of the intima, and subsequent giant cell and fibroblastic reactions (Figs. 20 and 21).

Coronary arteries, which mineralize early and then very rapidly lose such excessive minerals, can easily be distinguished from the normal coronary arteries, even by the tenth day of the experiments (Fig. 10). By this time, when von Kossa positive material has usually disappeared from the coronaries, the latter show a thickened intima which is hyaline and strongly P.A.S. positive (compare Figs. 11 and 12). Also metachromatic M.P.S.s accumulate in the perithelium. By the tenth or twelfth day the internal elastic membranes (normally prominent) have disappeared from previously mineralized coronary arteries. By the forty-third day, the walls of coronary arteries have thickened considerably, and the lumina have been narrowed as a result of the replacement of the walls by fibroblasts and the marked accumulation of P.A.S. positive and metachromatic polysaccharides (Figs. 13 and 14). Similar changes are observable, although in lesser degree, in medium-sized peripheral arteries, which do not seem to become mineralized even during the early phases of the experiments.

Some of the histologically demonstrable reactions of the aorta (to such severe but transient intoxication with calciferol) are depicted in Figs. 15 to 21. The extent of initial calcification of the ascending aorta is indicated in Fig. 15. In Figs. 16, 16*a* and 17 the disposition of von Kossa positive material in the thoracic and ascending aorta, respectively, are shown, 5 days after the last dose of calciferol. It can here be seen that mineralization occurs predominantly between and around the elastic membranes, the cores of the latter being visible as clear stripes within the black-staining mineral material (Fig. 16). Figs. 16 and 16*a* – high power views of serial sections of the calcified elastic membranes in the thoracic aorta – clearly reveal firstly, the intactness of the membranes (Fig. 16*a*) which are heavily coated with von Kossa positive material in Fig. 16; secondly, in Fig. 16*a*, the dark-staining metachromatic M.P.S.s accumulated around these intact, although calcified, membranes.

Serial sections of the ascending aorta (seen at low power in Fig. 15) are presented to demonstrate the following:

(1) The apparent thickening of the V.E.M.s by the peri- and intermembranous coating of minerals (Fig. 17. For apparent, as opposed to 'true', thickening of V.E.M.s compare with Figs. 18 and 19).

(2) The 'true' swelling of the collagen-like, paler cores and the diminution in the amount and 'pooling' of darker-staining fibrillar material around these swollen V.E.M.s (compare the lower part of Fig. 18 – 'normal cores' – with the middle portion – 'swollen cores' – in this specimen stained with Mallory's P.A.H.).

(3) In Fig. 19 the swelling of the V.E.M.s and fragmentation of the related reticulin fibrils, near the intima (upper Fig. 19), is clearly shown, as compared with the narrower (lower Fig. 19) V.E.M.s with their normal peri-membranous reticulum. The spiral arrangement of the latter is also indicated at *a* towards the lower left of this figure.

At 42 days after the last dose of calciferol residual patchy mineralization of the ascending aorta and fragmentation of the related E.M.s (towards the intima) is shown (Fig. 20). This mineralized part of the media and the overlying intima is undergoing fibrosis – i.e. developing into a localized scar. Associated with this latter change is an increase in fibroblasts and a marked accumulation of M.P.S.s (Fig. 21), comparable with that usually encountered in a healing cutaneous wound.

Thus, the morphological and tinctorial findings, in healthy blood vessels, showing that the V.E.M.s are not homogeneous in structure, are supported by the reactions induced in these membranes by injurious agents.

COMMENTS

It is possible to demonstrate that the vascular elastic membranes (V.E.M.s) in arteries are morphologically, tinctorially and histochemically distinguishable from the fine elastic fibrils in areolar connective tissue, pulmonary alveoli and elastic cartilages. It would seem that the 'core' of these V.E.M.s is maintained by a specialized alteration of the connective tissue components immediately related to these membranes. Reactions to injury, exemplified in rats by the metastatic mineralization following toxic doses of calciferol, indicate that the site of predilection for such mineralization is *not* the core of the V.E.M.s but rather their encasing reticulin and polysaccharide 'sleeves'. This sleeving of elastic membranes by polysaccharides and even by recognizable fibrils, here described, is in conformity with similar ground substance sleeves around corneal fibres described by van den Hooff (1952). The membranes themselves do not seem to be calcifiable, and in this respect resemble other collagen fibre bundles. On the other hand, the reticulin and polysaccharides coating the V.E.M.s, like similarly composed ground substance of bones and cartilages, seem especially liable to mineralization.

Taken together, the morphological and histophysiological evidence here presented, suggests that the integrity of the V.E.M.s is a function of the continuous activity of the surrounding connective tissue components, directed towards the maintenance of the membranes by the accretion and removal of molecules at their surfaces. This allows for constant regeneration of wear and tear trauma. The membranes themselves seem to act as 'organizers' for the metabolic activity of the surrounding polysaccharides and other connective tissue components, for, once these membranes are disrupted or destroyed, the connective tissues do not seem capable of replacing them easily. Such severe or prolonged injuries to the peri-membranous connective tissue are consequently succeeded by the usual reactions to injury, such as those seen in other parts of the body (e.g. in the skin), and characterized by M.P.S. accumulation,

followed by collagen deposition and scar formation, i.e. *repair*. Similar *repair* of arterial walls also occurs when the V.E.M.s are lysed in rats fed sweet-pea seed meal (Walker and Wirtschafter, 1956). The latter finding indicates that the M.P.S. accumulation, and other criteria of vascular repair here described, are not essentially a reaction to mineralization *per se*, but rather represent the counterpart, in arterial walls, of those repair processes, seen in cutaneous and other injuries, leading to scar formation.

The above data and suggestions also indicate that the most vulnerable portion of the V.E.M.s is the site of greatest metabolic turnover in the connective tissue polysaccharides – namely immediately around (sleeving) the elastic membranes themselves. This information may throw further light on the composition of the elastic membranes, the processes concerned in their maintenance, and may indicate new lines of investigation into the histochemistry of vascular diseases.

One fact that emerges clearly from the above data is that the M.P.S. accumulation, in severely injured arteries here described, and observable also in association with or preceding spontaneous or experimentally induced atherosclerotic lesions, merely represents one of the usual phases in the repair of all injuries in connective tissues – irrespective of their location in the body. M.P.S.s are capable of binding minerals and probably also lipids (see Wells, 1933, for review). Thus, the sequence of events in atherosclerosis, and in other arterial degenerations, may be as follows: disturbances in metabolic processes (and especially polysaccharide metabolism) at the surfaces of the V.E.M.s leads to failure in the maintenance of these membranes which consequently become damaged; such lesions may be, and usually are, followed by the accumulation (around the injured parts of the V.E.M.s) of muco- and other polysaccharides as part of the normal sequence of events in the healing of connective tissue injuries. The accumulation of M.P.S. in arteries (known to occur with ageing – Faber, 1949) provides a material basis for the binding of minerals, thus leading to calcification (as described by Lansing, 1955) or of lipid deposition (as in atherosclerosis) – depending on the prevailing metabolic conditions.

The disparity in susceptibility to degenerative lesions of various parts of the arterial tree may perhaps be accounted for on the basis of *different rates of polysaccharide turnover*. In our experiments it was clear that the heart and coronary vessels were damaged and mineralized

sooner than was the ascending aorta, which in turn was more susceptible than the thoracic aorta; the latter was again damaged sooner and more severely than the abdominal aorta. Thus in rats, intoxicated with calciferol, there seemed to be a distinct gradient of susceptibility of different parts of the arterial tree, resulting in a wave of injury, and then of recovery, both (injury and subsequent recovery) spreading from the myocardium and coronary arteries to the ascending, then the thoracic, pre-renal then post-renal sections of the aorta (in this order), and ultimately to the more peripheral vessels, like the femorals, carotids and mesenterics. Histochemical studies, thus far conducted, indicate diminishing concentrations of polysaccharide, proceeding from the coronaries towards the terminal parts of the arterial tree. All this evidence supports the above proposal to account for the well-known discrepancies in incidence of vascular degenerations along the vascular tree.

Understanding the genesis of vascular diseases would, if the above interpretations are correct, resolve itself into an analysis of those metabolic processes which are involved in the elaboration, by the ground substance and other connective tissue components, of vascular elastic membranes as well as of other fibres. A full appreciation of the basic processes involved both in regeneration and in repair is clearly essential. The experimental application of calciferol as well as of beta-aminopropionitrile (BAPN – the active principle of sweet-pea seed meal – see Strong, 1956, for review) offer unique opportunities for such studies on vessels, especially with the use of radioactive tracers.

SUMMARY

(1) Morphological, tinctorial and histochemical differences are described between vascular elastic membranes (V.E.M.s), pseudo-elastic fibres (P.E.F.s) and other elastic fibrils in connective tissues.

(2) These V.E.M.s seem to be comprised of central, possibly collagen-like, 'cores' coated or sleeved by reticulin and polysaccharides, and seem to be closely integrated with and dependent, for their integrity, on the activities of the surrounding connective tissues.

(3) Studies of degenerative vascular diseases, in rats and in man, indicate that severe or prolonged injuries lead to disruption of the cores of the V.E.M.s. This seems to be followed by disorganization

of those activities of the vascular connective tissues normally concerned in maintaining the integrity of the V.E.M.s. Such lesions progress through vascular *repair* (as opposed to regeneration) and culminate in fibrosis – the vascular equivalent of cutaneous scars.

(4) As in the repair of all injuries to connective tissues, severe enough to invoke repair, so too in vessels, one of the phases is characterized by that accumulation of mucopolysaccharides (M.P.S.s) which regularly precedes fibrosis.

(5) Mineralization, lipoidal changes or the development in vessels of pseudo-elastic fibres seem to be determined primarily on the severity, duration and nature of the disturbances in the peri-membranous connective tissue polysaccharide metabolism induced by the injury. This is indicated by the finding that experimentally induced mineralization of the V.E.M.s in rats first occurs *around* and not within these membranes. Only when the latter are disrupted does fibrosis, or the other changes mentioned above, supervene. The V.E.M.s thus seem to act, in some measure, as organizers of connective tissue metabolism in arteries.

(6) It is suggested that the varying susceptibility to injury of different parts of the vascular tree may be a function firstly, of the different rates of polysaccharide turnover in the peri-membrane connective tissues, secondly of the nature and severity of the injury, and thirdly of the prevailing metabolism.

(7) Most of the reactions to injury, observable in blood vessels, can be interpreted in terms of the usual histogenesis of the repair of injuries to connective tissues, no matter where they occur in the body. Such interpretations may be facilitated by the finer micro-anatomical details here outlined.

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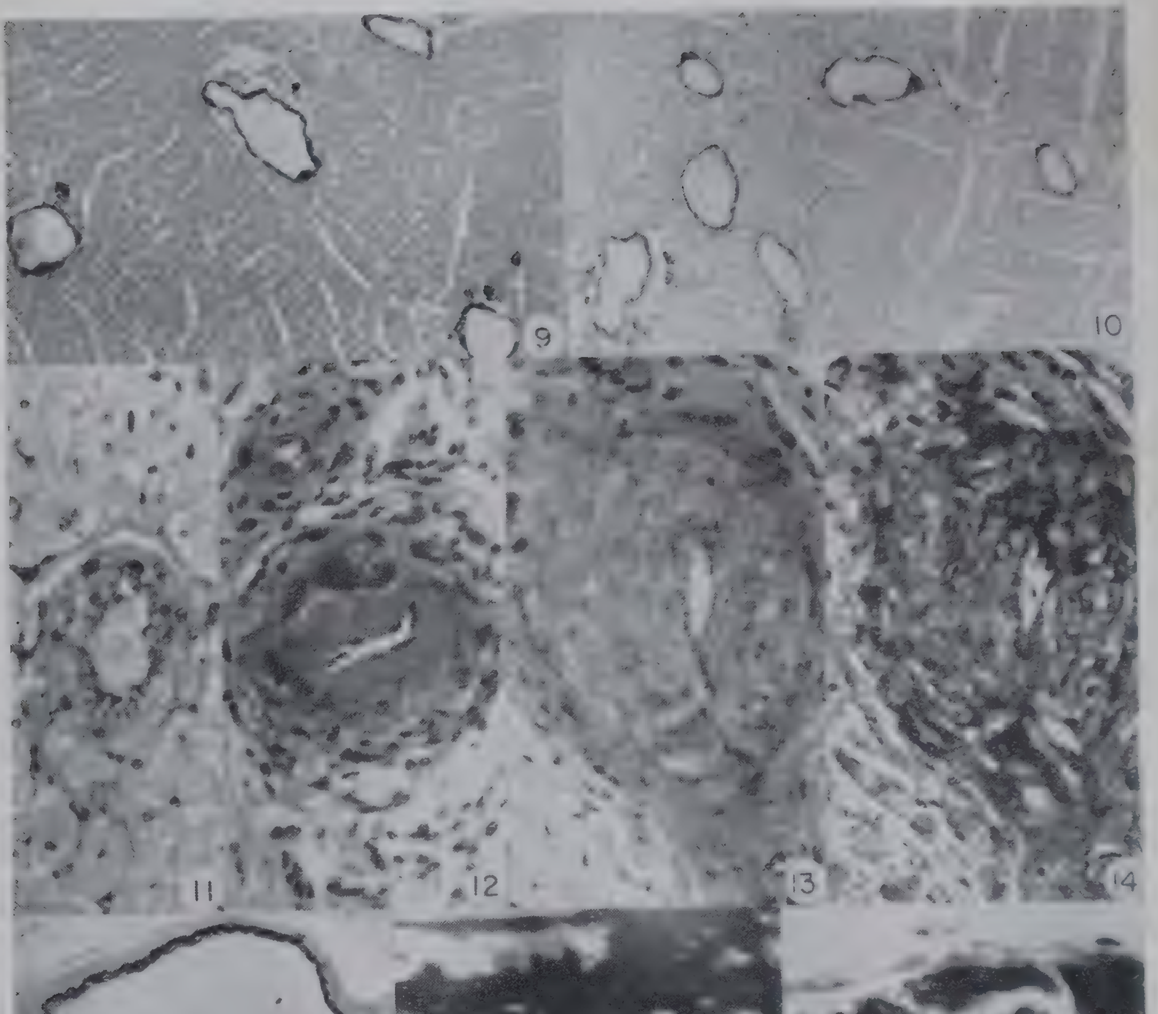
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PLATE II

FIG. 9

Heart and coronary arteries of a rat, one day after the last of five daily doses of 36,000 units of calciferol (i.e. sixth day of experiment), showing von Kossa positive walls of coronary vessels. Von Kossa preparation. × 50

FIG. 10

Same field as shown in Fig. 9, but several sections away, showing intense P.A.S. positive staining of entire walls of medium-sized coronary vessels. Periodic-Acid Schiff (P.A.S.) preparation. × 50

FIG. 11

Normal medium-sized coronary artery of a rat showing wavy, indented P.A.S. positive internal elastic membrane. P.A.S. stain. × 220

FIG. 12

Small coronary vessel in a rat 5 days after the last of five consecutive daily doses of 36,000 units of calciferol, to demonstrate intense broad P.A.S. positive, internal elastic membrane and deposition of P.A.S. positive material above and around the vessel. There is also an increased peri-vascular cellularity indicative of incipient fibrosis. P.A.S. method. × 220

FIG. 13

Markedly thickened cellular, fibrotic medium-sized coronary vessel in the heart of a rat 40 days after the last dose of calciferol. Note also the red-staining pools of P.A.S. positive material (grey in figure) towards 6 o'clock and 2 o'clock on this vessel. P.A.S. method. × 220

FIG. 14

Serial section to vessel shown in Fig. 13 demonstrating again marked fibrosis and pools of metachromatic mucopolysaccharide (especially well shown at 6 o'clock and between 12 and 2 o'clock on the walls of this vessel). Alcoholic toluidine blue only. × 220

FIG. 15

Extent of mineralization, confined to the intimal portion of the media of the ascending aorta in a rat one day after the last of five daily doses of 36,000 units of calciferol. Von Kossa method. × 23

FIG. 16

High power view of the thoracic aorta, from the same animal as shown in Fig. 15, to demonstrate the heavy mineralization around and between the elastic membranes. The central cores of the latter can be seen as paler non-mineralized streaks within the heavy von Kossa positive material. Von Kossa method. × 500

FIG. 16*a*

Serial section to Fig. 16 to show intact elastic membranes surrounded by darkly staining metachromatic polysaccharide which is also aggregated in pools around the uppermost elastic membrane in this figure. Alcoholic toluidine blue only. × 500

FIG. 17

Appearances of von Kossa preparation (same ascending aorta as shown in Fig. 15) to show distribution of von Kossa positive material in relation to wavy elastic membranes towards the inner aspect of the media. Von Kossa method. × 500

FIG. 18

Serial section to Fig. 17 to show thickened 'cores' of vascular elastic membranes towards intima, surrounded by pools or streaks of reticulin or elastin-like material; compare with healthy membranes and intermembranous fibrils below. Mallory's P.A.H. × 500

FIG. 19

Reticulin method applied to serial section to Fig. 18 demonstrating the thickening of the cores of the elastic membranes (towards top half of picture) and granular reticulin sheaths around these inner damaged membranes. The spiral nature of the reticulin fibres is also clearly shown around the apparently undamaged elastic membranes of the outer media (at *a*). Wilder reticulin preparation. × 500

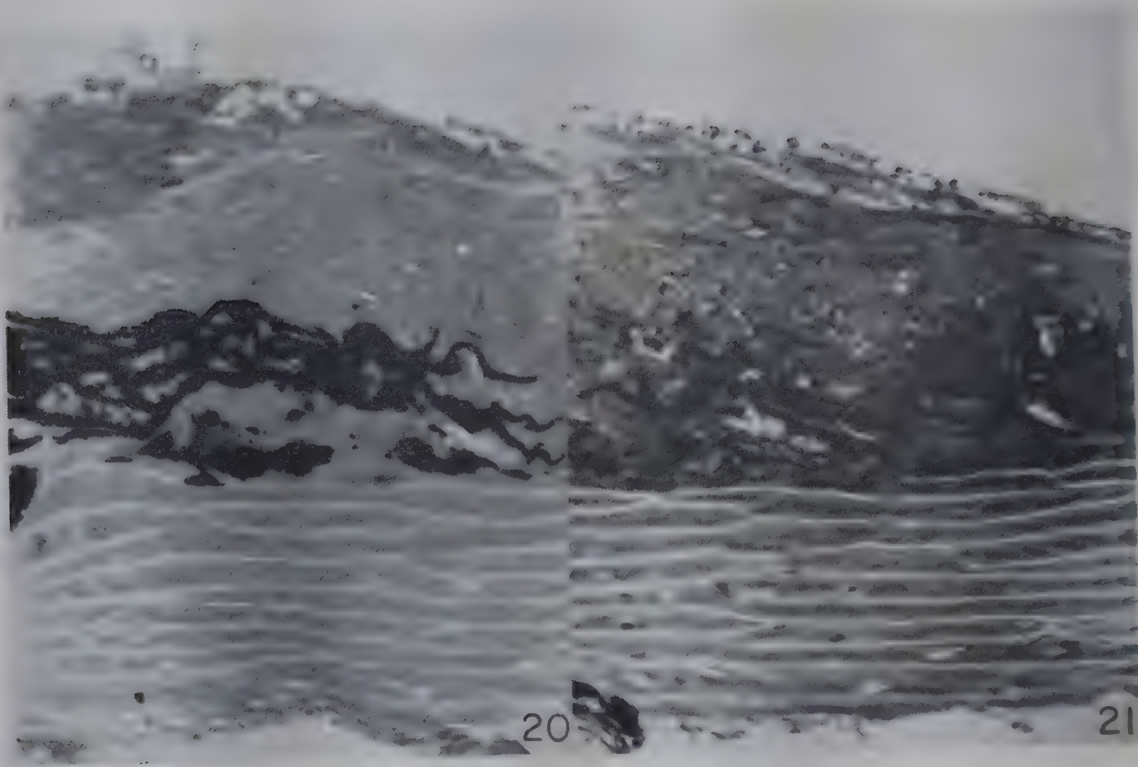

PLATE III

FIG. 20

A patch of residual calcification in the ascending aorta of a rat 40 days after the last of five doses of 36,000 units of calciferol. Note the fragmentation of the elastic membranes in the inner portion of media, surrounded by excessive minerals. The elastic membranes of the outer portion of the media are clearly shown to be intact. There has been fibrosis around this area of residual calcification with a plaque projecting towards the intima. Von Kossa preparation. × 150

FIG. 21

Serial section of same aorta as Fig. 20, to show massive deposition of metachromatic mucopolysaccharide (pools of dark-staining material at inner portion of media corresponding to areas shown as calcified in Fig. 20). The elastic membranes, in this damaged and fibrosing area, are clearly fragmented as compared with those in the outer portion of the media which are apparently still intact and undamaged. Alcoholic toluidine blue only. × 150

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GROUP DISCUSSION

DR. ROBB-SMITH could not agree with Dr. Gillman that elastic tissue in the dermis or elsewhere is practically never regenerated. It certainly takes a longer time than fibrous tissue but regeneration can occur and depends on satisfactory healing. In a 'good' scar, after about 40 days and onwards, one can see fine elastic tissue regenerating in the superficial dermis, although the pattern is never quite identical with the original subepidermal network. However, if there has been marked loss of tissue or inco-ordination between the epithelial and connective tissue regeneration as a result of infection, foreign bodies, etc., then elastic tissue regeneration was irregular or non-existent. He had confirmed these findings in experiments on rabbits, and there was no doubt that regeneration did occur although it was difficult to obtain and occurred slowly. He found Dr. Gillman's pseudo-elastic fibres of very great interest and agreed that they had not invoked the histopathological study they deserved. A congenital pathological condition in men, pseudo xanthoma elasticum involving elastic deposition in tissue and blood vessels, might be related to these abnormal elastic fibres. In relation to the alteration following deposition of calcium salts, he recalled that elastic tissue was one of the first tissues to take up injected colloidal solutions, for example trypan blue. This dye was later removed to other tissues, particularly the reticulo-endothelial system. Vitamin E deficiency in animals will reproduce similar aortic changes very nicely. The only point at issue with Dr. Gillman was the question of regeneration of elastic tissue.

DR. GILLMAN said that elastic tissue was not easily regenerated, at least not in scars. He had examined a wide range and number of scars in man and animals, the scar tissue varying in age from 2 days to 20 years, and even in very old scar tissue the elastic fibres which did reappear were abnormal, that is to say that, although elastic-like fibres reappear in *old* scars, they did not have a 'normal' morphology and did not acquire the original 'normal' pattern of arrangement. *In vivo*, trypan blue staining of

aorta seems to be due to dye accumulation in the 'sleeve' round the core of the aortic elastic membranes. In odoratism (sweet pea seed poisoning) fibrosis occurs only after rupture of the elastic membranes; this again indicating that damage of the elastic component may be an essential precursor of repair.

DR. BALÓ recalled the various forms of elastic regeneration as exemplified by reduplication of the internal elastic lamina in arteries in hypertension suggesting that elastic fibres can be regenerated.

DR. GILLMAN pointed out that he believes such 'reduplication' has been a misinterpretation by histologists. In reality, they were examining an accumulation of *pseudo*-elastic fibres, as defined by the methods and criteria outlined, rather than of new 'normal' elastic fibres. He wanted to stress the fact that calcification occurred not *in* but *around* the elastic membranes and can appear and disappear without fibrosis necessarily following. Large doses of calciferol which lead to severe or prolonged calcification followed by disruption of the elastic laminae were usually succeeded by foreign body reactions and fibrosis; in other words, repair, as opposed to regeneration.

DR. ASBOE-HANSEN asked whether elastic tissue regenerated in Cushing's striae or striae gravidarum in the skin which are normally devoid of elastin.

DR. GILLMAN said no, to his knowledge there was no reconstruction of the subepidermal elastic network of the skin in such striae. The depth of the injury seems to determine the type of subsequent reaction. Surface abrasions of epidermis were followed by complete regeneration of original architecture whereas wounds extending deep to the subepidermal elastic network always appeared to produce scar formation.

DR. BANGA mentioned that the sensitivity of elastic fibres to elastase increases with age. There appeared to be a relation between calcium content of the elastic fibres and their speed of dissolution in elastase.

DR. HALL said he had demonstrated that calcium is essential for the reaction between elastase and the elastic fibres. It seemed essential for the ions to be bound to the fibres. A large proportion of the calcium actually in the tissue will be present as calcium phosphate, but a small proportion must be bound to the tissue itself. It is quite conceivable that the precalcification triggers off the medial degeneration in arteriosclerosis.

DR. GILLMAN pointed out that after injury, several minerals including iron, seem to bind on to the peri-elastic 'sleeve' he had described. Such depositions do not occur in normal 'sleeves', no matter how high the blood calcium, but readily occur after injury.

DR. REED stated that several of Dr. Gillman's findings in aortic elastin were confirmed at the electron microscopic level. There were two different structural forms of aortic elastin: fibres and sheets. There was also a

close association with reticulin and collagen fibrils, whilst the elastic fibres were coated with dense amorphous material which probably represented mucopolysaccharides or a mucoprotein system. Had Dr. Gillman made any observations on local tissue pH values in the areas damaged by burns, ultraviolet light, etc.?

Dr. Gillman replied, no, he had not.

Dr. Hall stated that studies of dermal preparations from senile elastosis showed that the masses of material which appeared to be elastic fibres when stained and examined under the light microscope, proved to be degraded collagen when examined under the electron microscope. The pseudo-elastic fibres demonstrated by Dr. Gillman might similarly represent the products of a reaction stopped part way in the process of conversion of collagen to elastin. He believed that normally, collagen was degraded and then rebuilt to form elastin; but that under certain conditions, either through the lack of some intermediate or enzyme, the process was stopped half way. The conversion might be considered as having proceeded far enough to produce the tinctorial properties of elastic fibres, but not far enough to produce their physical properties.

Dr. Sylvén called attention to the high mass content of the elastic membranes, revealed by microradiography, as compared to the low mass of the surrounding connective tissue of the vascular walls. The mass distribution as such seemed to have bearings on the non-specific staining reactions described by Dr. Gillman, and might partly explain the microscopical appearance of the suggested 'core' structure.

Dr. Gillman stated that it would appear that the 'core' of these pseudo-elastic fibres possessed staining properties similar to those of collagen. There seemed to be a relation between four things in the skin and arteries; interfibrillar and intercellular substance, reticulin, collagen and elastic fibres. Disturbances of fibrillogenesis seemed to lead to the production of the abnormal structures which he had described.

Finally, Dr. Gross showed one lantern slide of fibres obtained from marine sponges. This demonstrated that two differently staining fibres could both be collagen as defined by X-ray diffraction, electron microscopy and chemical composition.

Dr. Gillman responded that this did not prove anything other than that Dr. Gross had yet another problem on his hands, namely to account for differences in arrangement of apparently identical electron microscopic fibrils, inter- and peri-fibrillar components responsible for the differences in staining reactions and morphology seen with the histologists' techniques. This would permit satisfactory progress from the presently known to the unknown.

ON THE NATURE OF THE METACHROMATIC GROUND SUBSTANCE POLYSACCHARIDES OF HEALING WOUNDS[1]

O. Snellman, R. Ottoson and B. Sylvén

The ground substance of healing wounds as well as other mesenchymal tissues exhibit during repair a marked increase in strongly metachromatic material, first observed by S. H. Bensley (1934) and later on independently described in more detail by Sylvén (1938, 1941). This material first appears in the ground substance about 24 hours after a clean incision is made. Maximum amounts of metachromasia are then observed at the height of fibroblast proliferation about the second or third day after incision. Later on, parallel with the formation of collagen fibres, a gradual decrease in the metachromasia of the ground substance as well as in the volume of ground substance materials as such is regularly noted.

Present knowledge is, however, limited as to the chemical composition of the metachromatic components of ground substance. The staining intensity and alcohol stability of metachromasia (cf. Sylvén, 1955) exceed that of hyaluronic acid and chondroitin sulphate, suggesting that heparin might constitute part of the polysaccharide components. Circumstantial evidence to this effect was obtained from observations on the changes on the part of the local tissue mast cells in the wound region. Most, if not all, mast cells seemed to become lysed and disappeared at about 24 to 36 hours after the wound was inflicted, e.g. at about the same time as the new metachromatic substance(s) just appeared in the same tissue region (for figures cf. Sylvén, 1941). This led to the hypothesis that the mast cells might deliver their metachromatic cytoplasmic material into the intercellular compartment during repair (Sylvén, 1941). Later on, it was claimed that the metachromatic material from 7 days' old wounds was largely digested by testicular hyaluronidase (Penney and Balfour, 1949), but this evidence may be equivocal.

[1] This study has been supported by grants from private donors, the Eli Lilly Comp. (separate grants to O.S. and B.S.), the Swedish Co-operative Women's Guild, and by institutional grants to the Cancer Research Division through a number of years from His late Majesty King Gustaf V's Jubilee Fund, the Cancer Society of Stockholm, and the Swedish Cancer Society, which is gratefully acknowledged.

Another possible explanation was that the fibroblasts might secrete the material under question. However, it would seem reasonable to expect some histochemical indication of such a secretion before the appearance of the metachromatic component, e.g. in the shape of some granular or other strongly metachromatic fibroblast material, and further to find an increasing concentration gradient around the first foci of growing fibroblasts. Such observations have, however, not been made, and therefore we are still inclined to assume that the mast cells may be implicated in this phenomenon.

To test this hypothesis, we planned to extract granulation tissue as a first step by means of salt solutions including histological control of tissue residues. Since the material was mainly located in the ground substance proper, it would seem possible to get it in solution before other polysaccharide material more closely bound to structural proteins. The KSCN extraction method was originally instigated for the selective extraction of the native heparin complex from the tissue mast cells (Julén, Snellman and Sylvén, 1950).

In other words, it was thought possible that the tissue mast cells might contribute to the polysaccharide components of the ground substance during repair. This did not mean that the mast cells would 'secrete the ground substance' as such, since it is realized that ground substance components are probably derived from several sources (cf. review by Sylvén, 1956).

MATERIALS AND METHODS

The control material embodies strips of pooled abdominal wall from adult albino rats, including the skin together with muscle and extraperitoneal fat tissue. Mast cells occur mainly in the skin proper, and no or very little metachromatic ground substance is found in this material. In the test series similar rats were subjected to laparatomy under sterile conditions, the mid-line incisions extending from the thorax nearly to the symphysis. On the third day after the operations, when the largest amounts of metachromatic ground substance and growing fibroblasts were expected, the healing wounds were cut out with scissors. The granulation tissue as such could not easily be isolated and consequently part of the surrounding skin and muscle had to be included. Infected cases were excluded. Pooled materials were kept frozen in solid CO_2 until further use.

The materials were finely ground in a steel mill together with solid CO_2 until a fine granulate was obtained, which was then extracted in the cold for 24 hours with a solution containing 0.5 M KSCN and 0.1 M potassium phosphate buffer at pH 7. Intermittent adjustment of pH to 7 was made. In this way a supernatant solution containing most of the heparin was obtained; the tissue residues were removed by centrifugation.

As previously reported (Snellman, Jensen and Sylvén, 1948) the KSCN solution extracts the metachromatic mast cell material almost quantitatively when used under strictly neutral conditions. pH shifts down to between 5 and 6 invariably resulted in precipitation or aggregation of the native heparin-polypeptide complex, provisionally regarded as microsomal in nature. The tissue residues from both materials mentioned above were fixed, embedded, sectioned and stained with Azure A and were devoid of metachromatic mast cell and ground substance materials. When such residues were treated by alkaline extraction according to the methods of Charles and Scott (1934), Wilander (1938) for the preparation of heparin, no heparin could be found by the anti-clotting assays. This suggested that the KSCN extraction had been quantitative, provided the anti-clotting assays were correct.

The proteins in the supernatant were precipitated by potassium phosphate at pH 7, added to saturation, and later removed by filtration. The solution then contained metachromatic substances together with some peptide material. In order to remove the latter and the salts and, at the same time, to concentrate the solution, this was ultrafiltrated through alundum tubes coated with nitrocellulose. This was a time-consuming treatment, which later on was changed for a more rapid process to be reported by Dr. Snellman.

The anti-clotting activity of the metachromatic materials obtained was assayed according to Jaques and Charles (1941). The carbohydrates in the solution were precipitated by adding absolute ethanol until a concentration of 70 per cent (vol.) was reached. The precipitate was redissolved and fractionated with ethanol.

Attempts to hydrolyse the material (6 N HCl) have been made and the hydrolysis products have been investigated by paper electrophoresis. So far no attempts have been made to investigate the content of hyaluronic acid and chondroitin sulphates simultaneously extracted, and no total sulphur determinations have been made.

RESULTS

The material precipitated at 70 per cent ethanol was determined. At such high alcohol concentrations some salts will also precipitate. The total yields obtained are therefore not very reliable. The yields of heparin in Table I have been calculated from the anti-clotting

TABLE I

	Normal Rat Skin		Granulation Tissue, 3 days old	
Experiment No.	*Total yield* in mg./kg. w.w.*	*Amount of Heparin in mg./kg. w.w.*	*Total yield* in mg./kg. w.w.*	*Amount of Heparin in mg./kg. w.w.*
1	257*	23.4	184*	7.7
2	285	54.3	146	5.6
3	146	47.3	160	1.8
4	149	22.9	240	1.2
5	49*	23.5	53	1.2
6	—	—	167*	10.0
	Average yields	35 mg./kg. w.w.		47 mg./kg. w.w.

* Including varying amounts of co-precipitated salts.

assays. All figures given in the table are calculated on a wet weight basis.

This did not seem very promising. Upon further consideration, however, the suggestion arose that some new material, appearing in the granulation tissue only, might have hampered the anti-clotting assays. Therefore it was decided to purify the materials further by ethanol fractionation (cf. Snellman, Jensen and Sylvén, 1949). Following the addition of ethanol up to a concentration of 50 per cent (vol.), a Fraction A was obtained, which contained all the metachromatic and anti-clotting material. Another Fraction B was precipitated by an ethanol concentration between 50 and 60 per cent. This substance was non-metachromatic and devoid of anti-clotting activity. The heparin content of Fraction A, determined by the anti-clotting assay, was as follows:

FRACTION A	from normal rat skin	35 mg. heparin per kg.w.w.
FRACTION A	from 3-days-old granulation tissue	36 mg. heparin per kg.w.w.

Hydrolysis of fraction A and subsequent paper electrophoresis in borate buffer at pH 8 showed only spots of glucuronic acid and glucosamine. Fraction B, amounting to about 60–80 mg./kg.w.w. of starting material showed after hydrolysis spots which could be referred to glucuronic acid and galactosamine. No protein residues were found.

Further, some anti-clotting experiments conclusively showed (1) that purified heparin (pharmaceutical) + substance 'B' (mentioned above) derived from normal rat skin, did not interfere with the anti-clotting activity of the heparin; but (2) that purified heparin + substance 'B' derived from the granulation tissue strongly inhibited the anti-clotting activity of the heparin. The nature of this peculiar material is still unknown. Fraction B contained so far only carbohydrates, and no trace of protein was detected by the biuret and ninhydrin reactions.

DISCUSSION

The results suggest that there is probably no difference in the total heparin content between the two tissue materials in question. Alkaline extractions performed with the more drastic method of Charles and Scott and of Wilander yielded similar amounts of heparin in both tissues as found by means of the KSCN extraction method. No new metachromatic substance was detected. It seems therefore that the previous assumption as to the occurrence of heparin in the intercellular medium of granulation tissue will obtain additional support. This will again call attention to the possible biological significance of heparin as a component of ground substance during fibroblast proliferation and perhaps also during the subsequent fibrogenesis. The mast cell polysaccharide thus released would be free to react with basic proteins and might influence a number of reactions in the growing cells. It could act as a sulphur transfer molecule, and further it might perhaps take part in the *in vivo* collagen organization in a similar way as indicated by the *in vitro* reconstitution experiments of Gross and his co-workers.

In the granulation tissue, the KSCN extraction method revealed an unknown carbohydrate inhibitor of the anti-clotting effect of heparin. The amounts so far obtained were, however, too small for a careful chemical analysis. Preliminary data suggest that perhaps a deranged chondroitin sulphuric acid might be involved.

GROUP DISCUSSION

DR. MEYER pointed out that an anti-clotting factor present in granulation tissue had recently been reported in the *Federation Proceedings*, 1956. He asked if sulphated polysaccharides other than heparin had been extracted from healing wound tissue.

DR. SYLVÉN replied that other acid polysaccharides, such as hyaluronic acid and chondroitin sulphate, no doubt were present, probably in increased amounts, but had so far not been extracted.

DR. MEYER said in his own work, on normal rat skin injected with ^{35}S sulphate there were two types of CSA, namely, B and C in comparable concentrations to that found in pig skin. The B has a negative rotation and contains chondrosamine but only the C fraction is hydrolysed by testicular hyaluronidase.

DR. SYLVÉN said they had until now used the thiocyanate method only aiming at the extraction and determination of the heparin content of the tissue, so he was unable to give any figures for the CSA content.

DR. GROSS inquired whether single or pooled rat skins had been used in this experiment.

DR. SYLVÉN answered that pooled albino rat skins from animals of the same age group, 2 to 3 months old and of the same weight, about 200 gm., were utilized; more than 1000 rats being consumed for this investigation.

DR. D. S. JACKSON said that on the evidence presented in his paper he would like to offer an alternative explanation of Dr. Sylvén's findings. By the methods used by Slack (*in press*) the presence of heparin was not noted, whereas there was a high concentration of other sulphated polysaccharides into which $^{35}SO_4$ was rapidly incorporated even in the early stages of development. The release of pre-existing heparin by the mast cell observed by Dr. Sylvén could be due to the trauma of incision. Possibly the true ground substance is formed later and hence the large uptake of $^{35}SO_4$. He emphasized that Dr. Sylvén's work was on wound healing in which a different mechanism may occur from that appertaining to the carageenin granuloma.

DR. SYLVÉN referred to the text and the diagram given in a previous paper of his (1941) and felt this explanation was surely refuted on the grounds of the sharp decrease in the number of tissue mast cells 24-36 hours after wound infliction and the most marked and coincident rise in metachromatic ground substance material in the identical tissue region.

DR. D. S. JACKSON said that this could still be an effect of the incision and wondered what was the explanation of the large uptake of sulphate into sulphated polysaccharide that occurred in the early stages of granulation tissue formation, since the heparin must presumably be already present in the mast cell before the wound was made.

DR. GROSS supported Dr. Jackson and mentioned experiments he performed on circular wounds in rat skin. The wound scab was very carefully removed from the surrounding skin and examined in the electron microscope twelve hours after wound infliction. This soft scab contained typical collagen fibrils which he believed had probably floated into the scab on the tide of fluid from outside the wound, so these substances demonstrated by Dr. Sylvén could be due to contamination from beyond the wound.

DR. ASBOE-HANSEN agreed entirely with the findings in rat skin. On the other hand, in rabbit skin wounds, both incised and punched, a marked increase in the number of mast cells could be observed within the metachromatic areas. He wondered whether Dr. Sylvén had related his curves to the tensile strength of the wounds and fibre formation.

DR. SYLVÉN had no data on the tensile strengths of those wounds.

DR. ROBB-SMITH wished to make two points; he felt that Dr. Gross's explanation was probably correct, as he himself had found a high proportion of collagen fibres in scab tissue. Secondly, he suggested that the sulphate uptake in healing wounds occurred in relation to the fibroblasts rather than mast cells.

DR. D. S. JACKSON agreed that this could be so.

DR. NEUBERGER questioned the relevance of this sulphate uptake. Could one assume that uptake necessarily meant molecular resynthesis? It was quite possible that one might get an exchange of sulphate groups in the side chains, but that did not necessarily denote synthesis.

DR. D. S. JACKSON quoted Dorfmann who showed that there was no detectable difference in the results following the uptake of ^{14}C-acetate, ^{14}C-glucose and $^{35}SO_4$.

DR. MEYER did not believe there was any disagreement between the turnover problem and the observations made by Dr. Sylvén. There was a known increased synthesis of hyaluronic acid and sulphated polysaccharide in granulation tissue. He wondered whether heparin acted as an intermediate, in transfer of sulphate to other acceptors such as tissue polysaccharides. He thought this was suggested by the ease with which sulphate can be removed from the NH_2 groups of heparin as well as the presence of curious mono-sulphated-N-acetylated fractions. Lippman found that his donor compound (adenine 3' phosphate 5' phosphosulphate) can catalyse the sulphation of a phenol, if the phenol has a higher energy than the acceptor. Heparin might well be such a catalyst.

DR. NEUBERGER observed that sulphate could be added on to a preformed non-sulphated chain.

DR. MEYER agreed and quoted the conditions in growing bone where very high uptake of inorganic sulphur occurs. The acceptor areas could be randomly distributed over the chain. There might be two different pro-

cesses in the formation of sulphated polysaccharides: chain formation and sulphation.

Dr. Sylvén pointed out the marked affinity of this ground substance material in healing wounds to the metachromatic polysaccharides. He had wondered long ago whether heparin could not have a role as a sulphur transfer substance, as it appeared that the heparin from mast cells was apparently very firmly bound to ground substance proteins in the loci of early granulation tissue formation. Heparin might also act as an enzyme-inhibitor (against RN'ase and other enzymes), and thus have a biological rôle during cell growth and synthesis of cytoplasmic proteins.

MORPHOLOGY AND DIFFERENTIATION OF THE CONNECTIVE TISSUE FIBRES

WILLY SCHWARZ

A connective tissue fibre, as identified under the ordinary microscope, is heterogeneous under the electron microscope. It consists of fibrils, which are generally invisible in the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. It is, therefore, necessary to distinguish clearly between the expressions 'fibre' and 'fibril'. The fibrils are the structural elements of a fibre. Thus, the expression 'fibril' may be made more precise, for histologists generally understand by fibril, a structure lying on the threshold of visibility in the ordinary microscope, and cannot say whether it is a fibril in the sense of an element or merely a thin fibre including both fibrils and the cementing substance. In histology based on the ordinary microscope one recognizes besides elastic fibres, two other types, namely, argyrophil and collagenous fibres. As electron microscopy reveals, both of the latter fibre-types have fibrils which can be characterized by the well-known complex periodic cross-striations. It has been suggested on the basis of this finding that histological differences between these fibre-types are conditioned, not by differences in their fibril-elements, but by the varying constitution of their cementing substances (Wassermann, 1956). For this reason, one speaks on principle of collagenous fibrils in connection with these periodic, transversely striated fibrils and regards the striation as the 'fingerprint' of collagenous fibrils (Gross, 1949, 1950). An argyrophil fibre consists accordingly of collagenous fibrils and a cementing substance typical for reticulin.

Since we assume the formation of fibrils to be basically extracellular, it is hard to imagine how *identical* fibrils are formed in different tissues and organs out of different cementing substances. Here we have to ask whether the observation of identical cross-striations is a sufficient criterion of identity of the fibrils. One could, for instance, interpret the cross-striations merely as a structural characteristic common to all connective tissue fibrils. In that case, the cross-striations would not be the trade-mark for collagen

alone, but simply for a connective tissue fibril. When one wants to differentiate connective tissue fibre-types by electron microscopy, one must proceed with the aid of other criteria as in histology. These can operate not only for the classification of individual connective tissue fibre-types in the various tissues and organs, they also need to be applied when one wishes to pursue the stages of development of ageing of one fibre-type.

One of these criteria is, of course, the varying mode of silvering of the connective tissue fibrils observed in the electron microscope picture. Here the method of connective tissue silvering described by Gömöri (1937), known from histology to be one of the simplest and most reliable, has proved best for the differentiation of collagenous and argyrophil fibres.

I should like to demonstrate the method of silvering collagenous fibrils with the example of plantar aponeurosis. Histologically this is a tendinous structure and one is dealing with collagenous fibres. When one isolates the collagenous fibrils by ultrasonic vibration, and then silvers them, most of the fibrils acquire silver particles in the region of the dark band.

The contrasting strengthening of the dark bands by the silver granules makes the transverse striation period of the fibrils appear more prominent (Fig. 1*c*). This finding was first described by Dettmer, Neckel and Ruska (1951) for tendon collagen of cattle, and then demonstrated by Schwarz (1953) for the sclera, by Probst and Ratzenhofer (1953) and Pahlke (1954) for human tendon, and by Linke (1955) for human skin. One could take the view that this involves, strictly speaking, not an implantation of silver particles in the fibrils, but rather a surface absorption of silver particles *on* the fibrils, a matter that cannot always be determined with absolute certainty when dealing with isolated fibrils. In order to clarify this question, I have also used the ultra-thin-section method (Fig. 1*b*). If the silver were deposited on the surface of the fibrils, longitudinal sections of collagenous fibrils would be free of silver unless a fibril surface lay directly at the level of sectioning. The described periodic silvering of the fibrils is, however, found uniformly throughout the section. One sees (Fig. 1*b*) the individual silver particles arranged in rows of four or five beside each other in the dark band. Therefore, one must speak of internal silvering of the collagenous fibrils. Irving and Tomlin (1954) do not believe in an internal silvering of the fibrils and ascribe the surface silvering which they found around

the fibrils to the polysaccharide in the coating of cementing substance. This finding could be demonstrated convincingly with their material (splenic reticulin), but unfortunately they did not use a tissue, the fibrils of which exhibit, under normal conditions, the internal silvering I have described. For this material the findings of Irving and Tomlin would require further testing, in which case the question would arise as to whether the polysaccharide within a fibril belongs to the cementing substance or whether it is to be regarded as a basic element of the fibril. Both views are conceivable. In the first case, one would have to regard the fibrils as impregnated by the cementing substance (in the sense described by Ranke). In the second case, one would have to assume that the polysaccharide is part of the structural framework of the collagenous fibril, as the findings of Grassmann (1955) suggest.

The method devised by Irving and Tomlin (1954) for silvering the argyrophil fibrils of the spleen had already been described by von Herrath and Dettmer (1951). Silver particles were found to be accumulated irregularly or in groups on the surface of the fibrils of argyrophil fibres. Fig. 2 shows fibrils from the arachnoid membrane that must be regarded as belonging to the group of reticular fibres. The latter lattice fibres exhibit in the adult organism a tendency for silver particles to accumulate on the surface of the dark band of the fibrils (Fig. 2*a*). This mode of silvering appears also in ultra-thin-sections (Fig. 2*b*); it is entirely different to the mode of silvering of collagenous fibrils. The silvering mechanism described by Irving and Tomlin is, in my opinion, entirely correct for these fibrils. This would imply that the fibrils themselves in an argyrophil fibre are argyrophobic, since the silver is deposited in the cementing substance. On the other hand the collagenous fibrils, with their internal silvering, must be regarded as argyrophilic.

It follows from the examples given that the varying methods for silvering fibrils can be used to distinguish between various types of connective tissue fibres. It was only a step further to apply the various methods to a study of the development of connective tissue fibres and their alterations throughout life (Schwarz, 1953; Pahlke, 1954; Linke, 1955; Jahnke, 1956). The fibrils of a collagenous fibre change their mode of silvering during development, as I wish to show in the case of the sclera. In the early embryonic stages, during which the sclera is incidentally still transparent, one finds only fibrils with an irregular external silvering (Fig. 3*a*). This mode of

silvering is encountered in all embryonic connective tissue forms, irrespective of the organ or tissue involved. In later embryonic stages the sclera becomes opaque. Bound up with this change is an alteration in the mode of silvering of a proportion of the fibrils. The silver particles arrange themselves on the fibril surface in groups (Fig. 3*b*). The periodic external silvering corresponds to the mode of silvering of reticular fibrils. As development advances the external silvering of most fibrils changes to the periodic internal silvering that is typical for collagenous fibrils (Fig. 3*c*). In this preparation of *sclera* from a 5-year-old child, one also finds isolated fibrils with periodic or non-periodic external silvering. This varying result of the mode of silvering of the fibrils in separate stages of development permits the conclusion that the fibrils are subject to qualitative changes during their development. The final stage of this qualitative maturation is the internally silvered fibril in the tendinous organs. Unless the electron microscopic findings are to be in direct opposition to ordinary microscopic findings, one may regard only the internally silvered fibrils as collagenous in the strict sense. In many organs this final stage of maturation is not reached. One finds throughout the life-span, fibrils which may be regarded as an intermediate stage in this process of maturation. Thus one finds in the cornea fibrils whose mode of silvering corresponds throughout life to the early embryonic fibril. The fibrils of the cardiac valve (Jahnke, 1956) display the same behaviour. The fibrils from the spleen (von Herrath and Dettmer, 1951), from the arachnoid membrane, and from the lung interstitial tissue (Schwarz, 1955) have a regular external silvering, corresponding to the late embryonic stages described for sclera. This group could be classified as reticular fibrils; likewise, from the point of view of the maturation process described above, they could be called pre-collagenous fibrils. This qualitative process is to be interpreted as a method of differentiation of the inter-cellular substance. One could draw a parallel between this process and the change in solubility of the collagen with advancing age (Banfield, 1954), or between this process and certain chemical differences of collagens and procollagens (Grassmann and Kühn, 1955).

In every organ the intercellular substance reaches a definite differentiation level which, under normal conditions, is maintained throughout life. This status of differentiation once reached and then maintained would appear to be different for every organ and cor-

responds to the functional character of the organ. The following examples show to what extent this correlation is true of a variety of tissues.

In human Achilles tendon, the internal silvering is complete (and hence the collagen fibrils fully mature) by the time the child learns to walk, indicating the early involvement of this tendon (Pahlke, 1954). On the other hand, an embryonic level of differentiation of the intercellular substance appears necessary for the function of the cornea (Schwarz, 1953) and this level is retained throughout life. Only under pathological conditions do the corneal fibrils differentiate further, the change being associated with the development of opacity of the cornea.

Another criterion, which permits a clear distinction of types of connective tissue fibres, is the evaluation of fibril-thickness. The increase in fibril-thickness is not necessarily synchronous with a change in mode of silvering (Schwarz, 1954; Lindner, 1954). One may regard the increase as a quantitative process of growth which must be separated from a qualitative process of maturation (differentiation). The distribution curve of fibril-thickness is characteristic for every organ. Fig. 4 shows the distribution curves of fibril-thickness for certain organs of adult individuals. The fibrils of cornea and cardiac valve reveal a minute range of fluctuation in thickness. Most of the counted fibrils vary from 20 to 30 mμ. in thickness. Conversely, the tendinous connective tissue organs exhibit a very wide range of fluctuation in fibril-thickness and it is possible to distinguish between various members of this group on a size distribution basis. In this fashion it is even possible to distinguish between palmar and plantar aponeurosis which are histologically identical. The interstitial connective tissue of the lung, the skin and the arachnoid membrane are intermediate in their distribution curves between bradytrophic tissues (cornea, cartilage, cardiac valve) on the one hand, and tendinous organs on the other.

When one considers the distribution curves of the various organs during their development, one discovers the following: in the early stages of development of a connective tissue there is always a distribution curve of fibril-thickness corresponding approximately to that of the cornea. In the latter tissue one always finds extremely thin fibrils and a narrow range of fluctuation in fibril thickness. While the corneal fibrils and the fibrils of other bradytrophic tissues maintain this status throughout life, the connective tissue fibrils of other

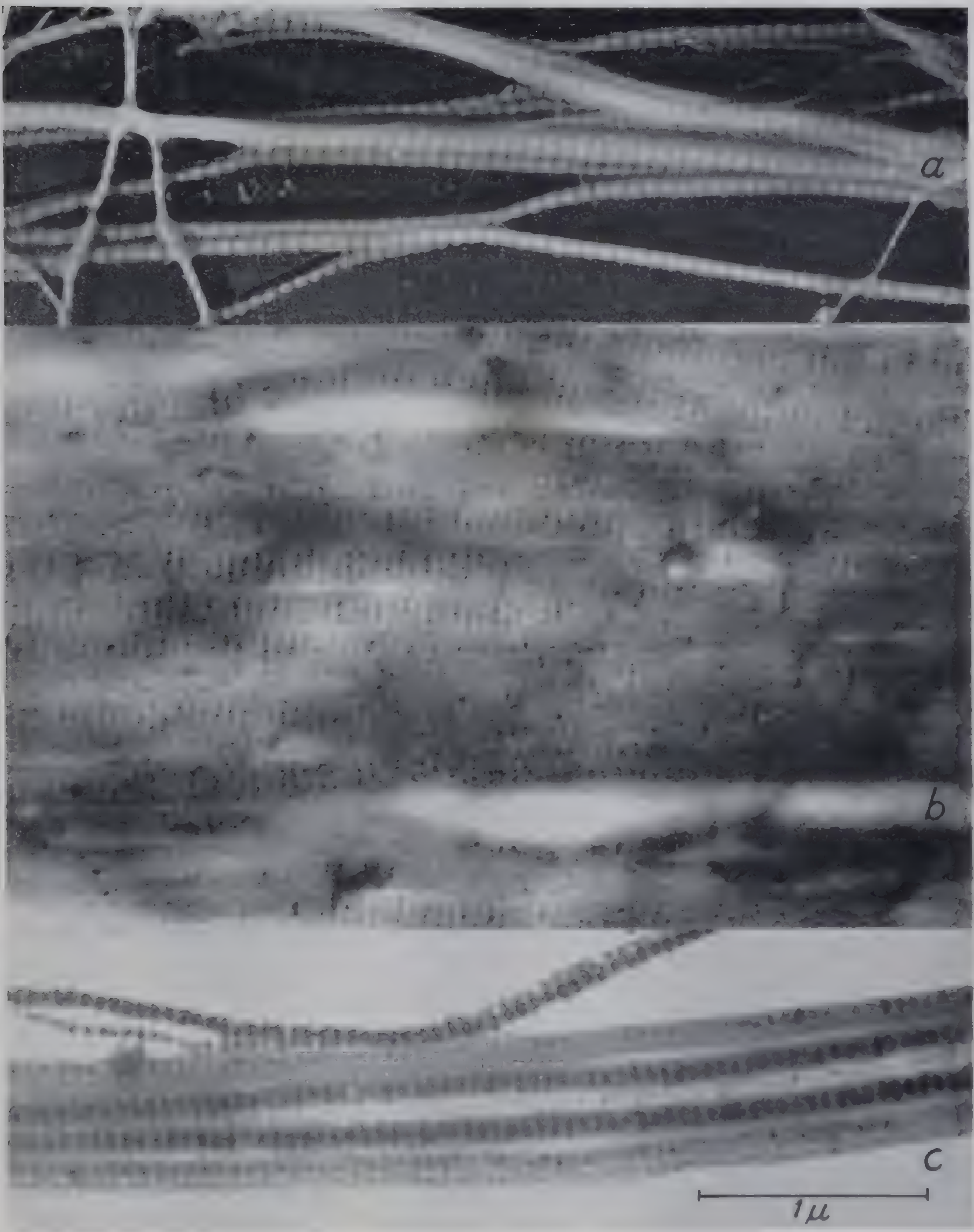

Fig. 1

Plantar aponeurosis in a 68-year-old man. × 25,000

(a) Fibrils isolated by means of high-frequency resonance. Shadowed with palladium.

(b) Longitudinal section of a fibre. The material was silvered according to Gömöri's method before the implantation.

(c) Fibrils isolated by means of high-frequency resonance. After the application of resonance, silvering according to Gömöri's method.

FIG. 2
Arachnoid membrane of a 30-year-old woman. × 25,000
(*a*) The same preliminary treatment as in Fig. 1*a*. Shadowed with palladium.
(*b*) Section of several fibres. The material was silvered according to Gömöri's method before the implantation.
(*c*) Silvered fibrils; same preliminary treatment as in Fig. 1*c*.

FIG. 3
Various stages of development of sclera. × 1,8000
The fibrils were isolated by means of high-frequency resonance and then silvered according to Gömöri's method.
(*a*) Fibrils from the sclera of an embryo 4 cm. long.
(*b*) Fibrils from the sclera of an embryo 16.5 cm. long.
(*c*) Fibrils from the sclera of a 5-year-old child.

organs are subject to a growth process (Gross, 1950; Schwarz, 1953; Pahlke, 1954; Linke, 1955). This process of growth is different for every organ, just like the differentiation process described above. When one compares the development of distribution curves for a tendinous organ (Fig. 5) with the composite distribution curves of

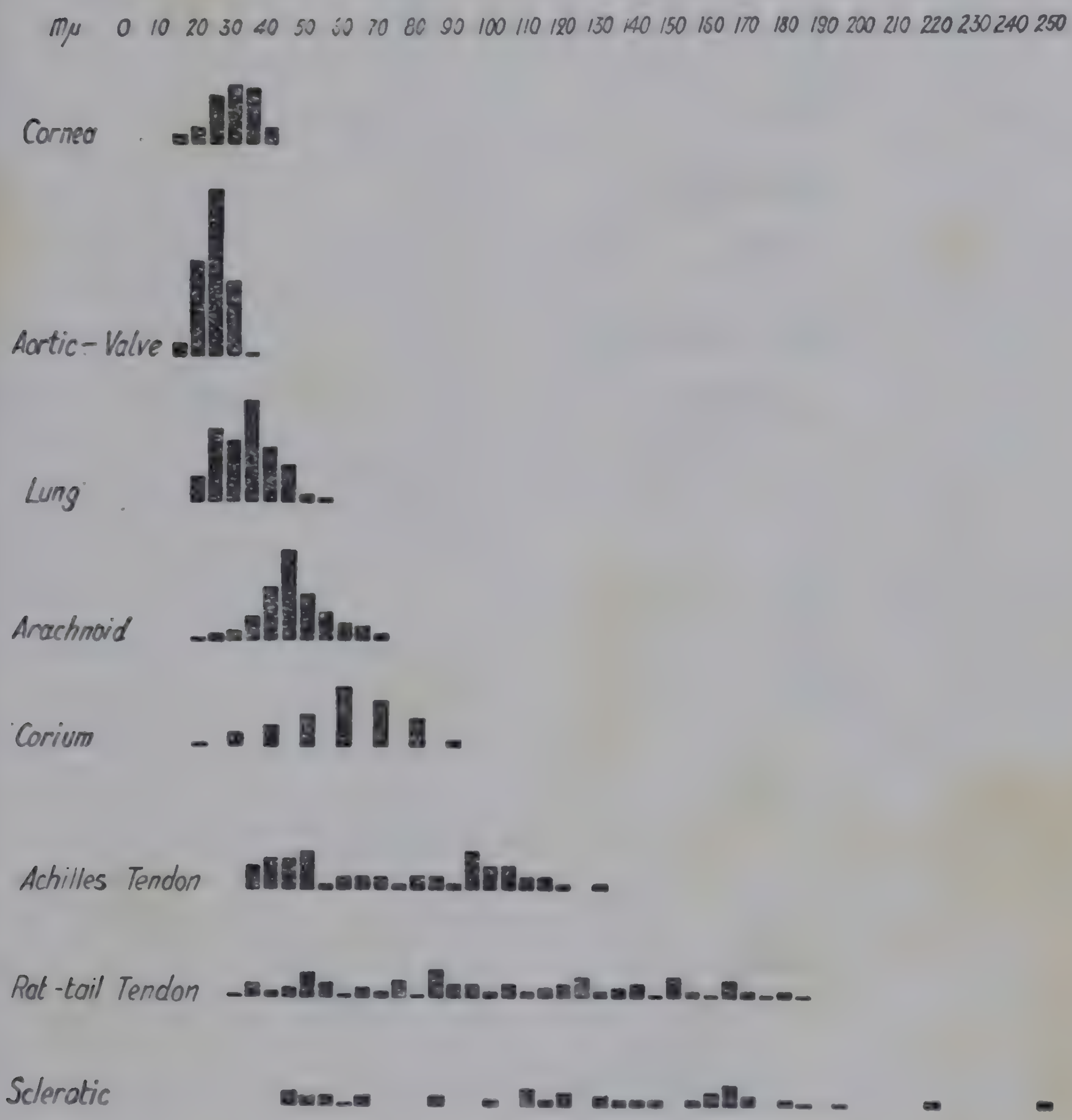

FIG. 4
Distribution-curves showing fibril-thickness of various intercellular substances, from connective tissue, from adult individuals. 200 fibrils were counted out in each case.

various adult organs (Fig. 4), one finds the following results: the development of the Achilles tendon or of the sclera comprises a spectrum of distribution curves at the beginning of which stands the early embryonic stage with the thinnest fibrils and narrowest range of fluctuation, and a final stage indicated by a widely disparate

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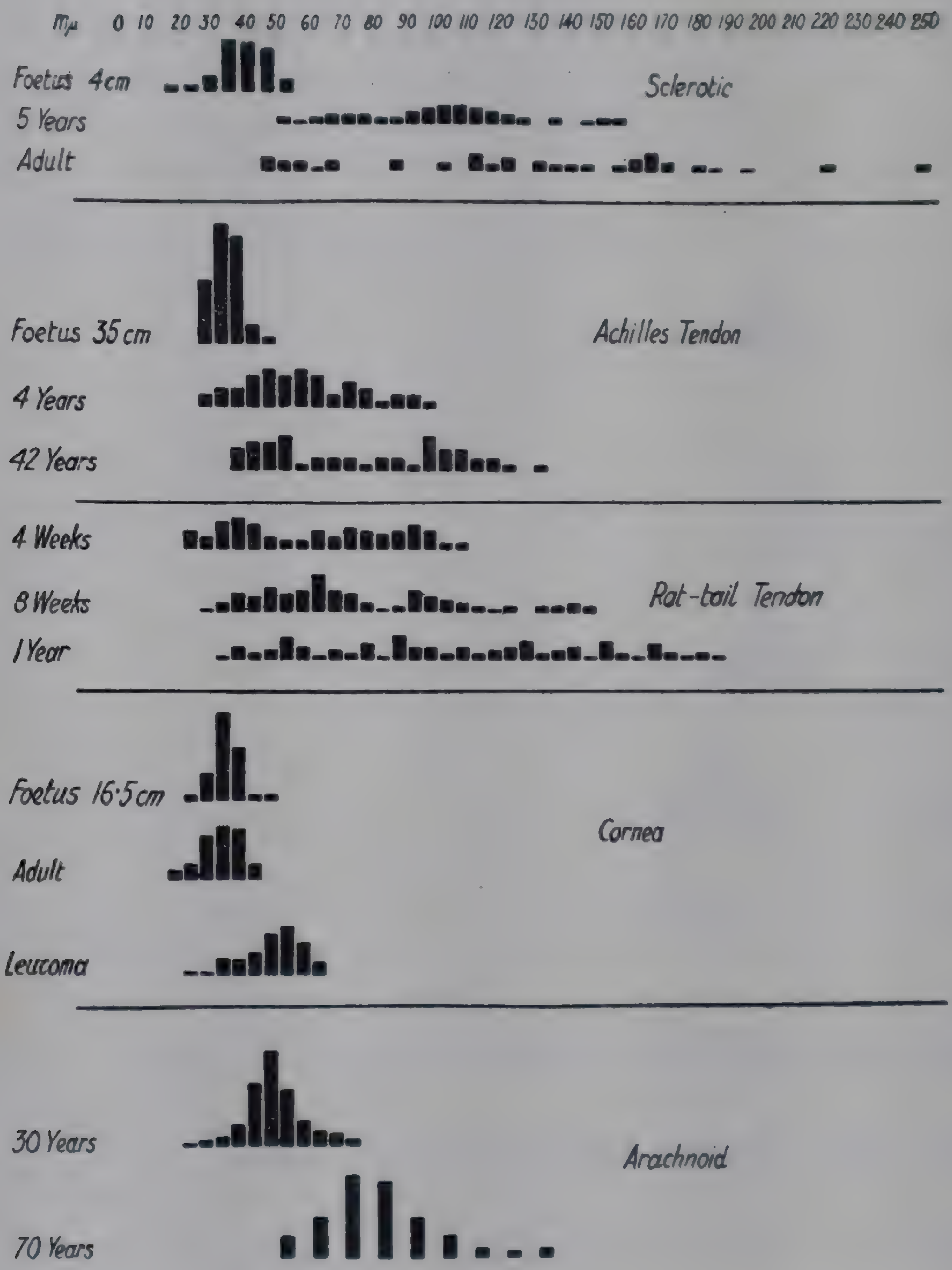


FIG. 5
Distribution-curves showing fibril-thickness of several connective tissue-forms during their development.

distribution curve. We find a similar spectrum when the various connective tissue organs are arranged according to the tendency of their fibrils towards growth (Fig. 5).

This tendency towards growth is, like the process of differentiation, obviously regulated by the individual organs. Both growth

and differentiation are stopped at that point in time when the functional optimum of the respective intercellular substance is reached. In this way, for each organ a specific status of intercellular substance is reached which is appropriate to the function of the organ. A change in the state of the intercellular substance is possible in various ways. In the case of intercellular substances whose fibrils do not reach, under normal conditions, the final stage of differentiation and growth, characterized by thick, internally silvered collagenous fibrils, further growth and differentiation in the direction of collagen are possible only under pathological conditions or in the event of senile alteration. An example of this is the corneal cicatrix (leucoma) whose distribution curve is presented in Fig. 5. Here one finds a further differentiation and also a growth of the fibrils (in comparison with the arachnoid membrane of a 30-year-old) due to senile changes. The possibility of further growth and differentiation of the fibrils is not present in those organs in which most of the fibrils have under normal conditions already arrived at the final stage. Here only regressive processes can lead to changes in the fibrils. As an example of this one might mention the skin whose fibrils grow thinner again in old age (Linke, 1955). But alongside this regression, there also occurs in the thick fibrils, in advanced age a certain degree of production of new intercellular substance and from this fresh formation of thin fibrils. Because of this, the range of fluctuation remains relatively great even in old age.

All of these senile or pathological changes, regardless of whether they consist of further differentiation producing increased thickness or regressive changes in those tissues incapable of further differentiation, impair the functioning of the tissue concerned.

Thus far I have kept the fibrils and their changes in the foreground of attention. But the second component of a connective tissue fibre, the cementing substance, is also subject to qualitative and quantitative changes. These changes are, however, difficult to understand with existing morphological methods, although a study of shadowed pictures of intercellular substance offers more hope of success. Here one can see, after appropriate preliminary treatment, a varying mass of fine coarsely granular material alongside the fibrils. This substance appears to be partly dried up, and does not completely surround the fibrils. One finds a considerable mass of cementing substance in embryonic, arrested tissues or at embryonic differentiation levels of such tissues. As the fibrils grow and differentiate, the rela-

tive mass of cementing substance generally decreases. In the thick and differentiated fibrils of the tendon only traces of cementing substance are found between the fibrils. Only in the sclera is a noticeable amount of cementing substance present and this may have undergone a considerable degree of differentiation. As well as giving an idea of the amount of cementing substance, the electron microscope picture enables one to estimate roughly the affinity of the cementing substance for the fibrils. The affinity is reduced as differentiation progresses. In this connection I cite Fig. 1*a* and Fig. 2*a*. After identical preliminary treatment of both preparations, a difference can be seen, not only in the amount of cementing substance, but also in the way in which it adheres to the fibrils. The fibrils of plantar aponeurosis (Fig. 1*a*) lack a coating of cementing substance, most of this material lying free and dried up between the fibrils. The fibrils of the arachnoid membrane, on the other hand, show a distinct coating of cementing substance partly concealing their periodic cross-striations. Owing to this large amount of cementing substance, the fibre complex is much better preserved than in the plantar aponeurosis. Various connective tissue fibres can thus be distinguished by the evaluation of shadowed fibril-preparations.

Another means of distinguishing is available in that one can estimate the polysaccharide content of the cementing substance electron microscopically. To this end, one can employ enzymic methods (hyaluronidase) together with the periodic acid-silver reaction (Dettmer and Schwarz, 1954). Thus it can be shown that the polysaccharide content of the cementing substance is highest in embryonic tissue and that it falls as differentiation proceeds.

In a cross section of the connective tissue even when the cementing substance cannot be recognized, the fibres can be distinguished, and the fibril-thicknesses, the topographical relationships of fibrils to each other and the thickness of fibres are all susceptible to evaluation. In a mature collagen (Fig. 1*b*) the fibrils lie close to each other, whereas the fibrils of the arachnoid membrane (Fig. 2*b*) appear to be loosely enclosed within the fibre. One must, however, imagine the spaces between the fibrils to be filled with cementing substance. Apparently the cementing substances are concealed by the impregnation material which remains in the section.

The identification of elastic fibres by means of the electron microscope is not difficult. They are easy to recognize in a section as

homogeneous, ramified or unramified bands which reveal no fibrillar structure under normal conditions. After extended 'fixation' in buffered osmic acid, the homogeneous appearance of the elastic fibre disappears, and then a net of filaments 70–100 Å thick appears. These filaments might be regarded as the proelastin which Hall and associates described (1952). The elastomucin appears to go into solution in buffered osmic acid (Dettmer, 1956). Further detailed observations are needed to determine whether the elastic fibres undergo a diminution of growth and of differentiation processes as do the other connective tissue fibres. Our experiments on the aorta are not yet finished.

I have tried to present the differences in connective tissue fibres which are observed with the electron microscope. The growth and differentiation of the intercellular components of connective tissue, on the evidence submitted would indicate the existence of a dynamic system, the conditioning factors for which are unknown to us. Research on these factors will only be possible when the cells are included in the treatment of the problem.

SUMMARY

Argyrophil and collagenous fibres consist of fibrils which are often invisible under the ordinary microscope, and of a cementing substance which occupies the spaces between the fibrils. The fibrils of both fibre-types display the well-known periodic cross-striation. Thus, they present the same appearance under the electron microscope. Most investigators regard this cross-striation as the 'finger-print' for collagen; a precise distinction on this basis between the two fibre-types is not possible, however, with the electron microscope. Fibre thickness has, therefore, been adopted as the criterion for distinguishing reticular from collagenous fibrils, in order to establish a connection with histological observations. The increase in thickness signifies only a quantitative process and may be regarded as evidence of growth of the fibrils. The method of Gömöri for silver-impregnation of the connective tissue, however, enables the electron microscopist to differentiate argyrophil from collagenous fibrils and fibres qualitatively. The collagenous fibrils, of the plantar aponeurosis for example, take up silver on the dark band of the cross-striation, thereby making the striations more prominent. This internal silvering is apparent not only on isolated fibrils but in

sectioned material as well. On a longitudinal section of a fibril 180 mμ. thick, one sees four to five granules lying in a row beside each other in the dark band of the fibril. On the other hand, in the case of argyrophil fibres the silver is found on the surface of the fibrils. The silver granules are arranged in groups which correspond topographically to the dark bands of the fibrils. Thus the reticular fibril is characterized by a periodic surface silvering. The actual fibrils of the argyrophil fibres are argyrophobic and the fibrils of the collagenous fibres argyrophilic. A qualitative distinction between different fibrils and between the fibres is possible through the utilization of this characteristic affinity for silver. The internally silvered collagenous fibrils are first found in the connective tissue of the organs in question at a certain stage of development. Observations on the development of such connective tissue (sclera, Achilles tendon) reveal a differentiation which is visible in the electron microscope. The fibrils of the embryonic sclera show a completely random silvering on their surface. This manner of silvering is conditioned by the fibrils' coating of cementing substance. In the course of further development of the tissue, the silver granules arrange themselves on the surface of the fibrils in groups which correspond topographically to the reticulin of the histologist. As the differentiation proceeds the silver granules are increasingly impregnated in the dark band of the fibril. The silvering depends probably on carbohydrates. This process of differentiation takes place with all embryonic and/or newly formed fibrils, but all connective tissues do not reach the final stage (internal silvering). On the contrary, many tissues remain at an intermediate stage of the differentiation (reticular connective tissue, interstitial tissue of the lung). The reticular stage is in fact never reached by bradytrophic tissue of the cornea during normal development. A further differentiation, beyond that which is typical for each connective tissue, signifies an impairment of function. For example, the cornea displays a further differentiation of the intercellular substance which is bound up with loss of transparency as in leucoma. In the parenchymatous organs, a further differentiation of the interstitial tissue implies a change which we know as sclerosis. The cementing substance, which is found between the fibrils, participates in the differentiation process of the fibre. Its relative amount decreases during the process of differentiation. Its quality changes also, even though this is difficult to interpret morphologically. One must, for genetic reasons, regard the

fibre forms of the reticulin and collagen as an extended spectrum, in which the reticular and collagenous fibre signifies only a roughly schematic classification of fibre forms. Morphologically the elastic fibre assumes an exceptional position. It consists of filaments and cementing substance. The elastic fibres appear homogeneous in section inasmuch as the filaments are entirely masked by the cementing substance. These filaments may, however, be demonstrated by using special methods, but more exhaustive observations are needed to determine whether the elastic fibres go through a process of differentiation similar to that of the other connective tissues.

GROUP DISCUSSION

Dr. Banga asked whether silver binding depends on the histidine content of collagen as described by Dr. Grassmann. Dr. Orekhovitch had stated that procollagen contained three times more histidine than native collagen. She had also found that the silver binding of procollagen was three times more than that found in native collagen fibrils. After extraction of mucopolysaccharide from the native collagen she found that the silver-binding capacity of the fibres dropped also. Potassium iodide relaxed fibrils (metacollagen) have a much smaller silver-binding capacity than the native fibre. Does silver binding depend on the mucopolysaccharide or the histidine content of the fibril?

Dr. Grassmann replied that the silver binding he described in 1951 and the silver staining described here today were two quite different things. One is a complex binding of the silver ions and the other is a deposition of silver metal particles. They had found a stoichiometric correlation between the histidine content of collagen and the quantity of silver ions bound by collagen. They had found exactly the same histidine content in procollagen as in native collagen. In two samples of collagen (purified by salt extraction and followed by treatment with calcium hydroxide and trypsin) the percentage of histidine-N of total nitrogen obtained was 1.52 per cent and 1.48 per cent. In two samples of procollagen the percentages of nitrogen obtained were 1.35 and 1.41. The use of stereoscopic electron microscopic pictures can distinguish whether particles of silver lie inside or outside small collagen or reticulin fibrils. Work had already been started on Achilles tendon and long-spacing fibrils and they had demonstrated quite definitely that some silver particles lie within the fibril.

Dr. Schwarz felt that most histological stains were non-specific and that of all the various silver impregnations, the Gömöri method proved

best for collagen under the electron microscope. Like Dr. van den Hooff, he was convinced that washing time had to be restricted to 10 seconds but he had managed to do it in a test tube, using a centrifuge.

In answer to Dr. Meyer, Dr. Schwarz said that the cornea contained no elastic fibres. It was a specialized form of connective tissue with thin fibrils and a lot of cementing substance which masked the fibrils, thus making the translucency. If the fibrils became thicker the relationship to the masking cement substance was disturbed and leucoma appeared. The cementing substance may be in a different form in the cornea. A twelve-day corneal graft shows swelling and depolymerization. The cornea takes up silver in an irregular manner like an embryonic tissue (bradytrophic tissue).

Dr. Reed pointed out that the wide differences in fibril width and silver-staining properties that Dr. Schwarz had demonstrated seemed to be accompanied also by a wide difference in their physical and chemical properties in this reticulin-collagen system. For instance, they all shrank and responded to different enzymes at different rates and he thought it might be possible that, when the biochemist was extracting material from this system, he was dealing with fibres that differed both in age and chemical composition.

Dr. Robb-Smith said that although agreeing with Dr. Schwarz as to empiricism and plurality of silver-staining methods, he considered that the Gömöri technique was not necessarily the best but that the best method was the one that worked best in any particular laboratory.

Dr. Gross pointed out the difficulty of taking electron micrographs of gold and silver. The electron beam caused crystallization and aggregation of these metal particles so that their configuration could depend on the time the specimen had been in the electron microscope.

Dr. Partridge asked if it were possible that the production of silver grains in silver-stained material was similar to the photographic process which is catalysed by sulphur compounds and reducing substances of low molecular weight. If that were so, it was possible that the differences of various fibres from different tissues might be due to the presence or absence of these reducing agents near the fibres. It would then be unnecessary to assume that the collagen varied in itself from place to place.

Dr. Schwarz replied that the size of the silver granules depends on the site of deposition. As to relation to photographic process, there are certain parallels, though the processes are not exactly identical. In reply to Dr. Gross's objection, Dr. Schwarz answered that he had taken precautions mainly consisting in keeping the beam intensity low.

ELECTRON MICROSCOPE AND CHEMICAL STUDIES OF THE CARBOHYDRATE GROUPS OF COLLAGEN

W. GRASSMANN, U. HOFMANN, K. KÜHN, H. HÖRMANN, H. ENDRES AND K. WOLF

The opinion that carbohydrate components, which are known to be elements of the ground substance of connective tissue, play an essential role in the formation and in the structure of collagenous fibrils, is based on the following findings:

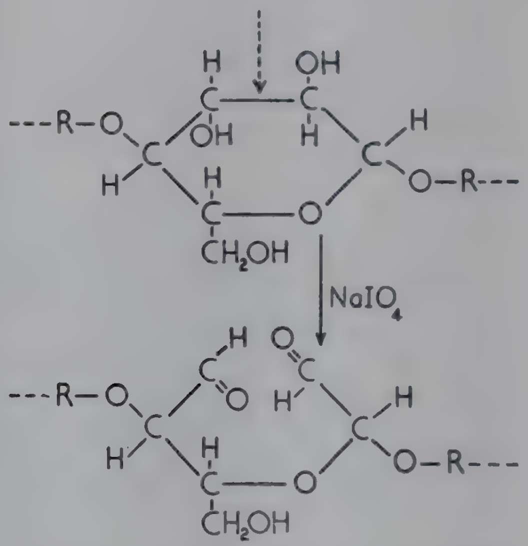
(1) The customary standard methods for the analysis of glycoproteins such as, for example, the orcinol reaction (Sørensen and Haugaard, 1933), the anthrone reaction (Dreywood, 1946), the Elson-Morgan reaction (Elson and Morgan, 1933; Blix, 1948; Boas, 1953), etc., have shown that all collagenous substances contain small amounts – not more than 1 per cent – of carbohydrates (Grassmann and Schleich, 1935; Beck, 1941; Bangle and Alford, 1954; Moss, 1955). Such carbohydrates were isolated and identified by means of chromatographic methods, and the following were described as components of collagen: glucose (Grassmann and Schleich, 1935), galactose (Grassmann and Schleich, 1935), mannose (Gross, Highberger and Schmitt, 1952), fucose (Glegg, Eidinger and Leblond, 1953), glucosamine (Schneider, 1940).

(2) It has further been shown that the formation of some distinct types of fibrils in collagen solutions is caused and controlled by small amounts of accompanying substances containing acidic carbohydrates. The presence of hyaluronic acid, α_2-glycoprotein or heparin results in the formation of 'long-spacing collagen' (Highberger, Gross and Schmitt, 1951), whereas nucleic acid or ATP form 'long-spacing segmented collagen' (Schmitt, Gross and Highberger, 1953). The presence or absence of such carbohydrate components also seems to be an essential factor for the solubility of collagen, in particular its solubility in diluted acids (Jackson, 1953).

(3) Gömöri's silver staining (Gömöri, 1937) which is known from histology, the PAS reaction (McManus, 1946), and the periodate

silver reaction (Dettmer and Schwarz, 1952/53) are apparently carbohydrate reactions. The two latter ones are certainly due to aldehyde groups formed by the action of sodium periodate.

FORMULA I



These results were further pursued in two directions: (*a*) Schwarz and Dettmer (Schwarz, 1955; Dettmer, 1955) have shown that the silver staining of collagen, in particular the method employing periodate-silver urotropin (Dettmer and Schwarz, 1952/53), can be used to trace carbohydrates in the electron microscope or – to put it more cautiously – to trace substances that react to sodium periodate by forming aldehydes. Pahlke (1954), by using this method, was able to show that the deposits of silver grains in embryonic sinews occur mainly in the amorphous ground substance. With increasing age these silver deposits in the interstitial substance gradually decrease and the silver grains are found in fine dispersion on the surface of the fibrils, and in adults such deposits occur in a regular order in the dark bands of the cross striation. According to Schwarz and Pahlke this periodic 'intrafibrillic silver staining' indicates the maturity of a fibril. (*b*) In our laboratory, on the other hand, we were able to show (Grassmann and Kühn, 1955; Grassmann, Hörmann and Klenk, not published) that the various kinds of collagens are dissolved and disintegrated if treated with sodium periodate or similar reagents for some length of time. In this process, α-amino groups of glycine, alanine, valine and no others are set free. Their quantity is equivalent to an average chain length of about 24

amino acids in the case of the disintegration product obtained from collagen and of 18 amino acids in the case of procollagen.

We have studied the dissolution of collagen fibrils under the action of periodate through the electron microscope by employing the silver-staining method (Kühn, Hofmann and Grassmann, 1956). Figs. 1, 2, 3, 4 show the action of a 4 per cent sodium periodate solution on Achilles tendon fibril in four subsequent stages, namely after a quarter of an hour, after one hour and a half, after 18 hours, and after 43 hours, whereupon, in each case, the silver-staining method was employed in the usual manner. Fig. 1 shows a normal order of silver deposits in a collagenous fibril after 15 minutes' treatment with periodate.

Even after only one hour and a half (Fig. 2) a splitting off of fibril parts can be observed. The silver deposits still give the impression of being fairly periodic. The longer the oxidation process went on, the darker the silver solution became, which again indicates that part of the aldehyde-bearing material dissolves and forms silver grains in the solution. After 18 hours (Fig. 3) the silver grains are found to have become progressively smaller in size but are still in periodic order. It is remarkable that now, under the relatively thinner silver deposits, the normal cross striation of the fibril begins to show, but because of the lack of any phosphotungstic staining such cross striation is not sufficiently contrasty in these experiments. After 43 hours (Fig. 4) the major parts of fibrillic substance are dissolved. The few remaining parts, however, still reveal periodic silver deposits.

Procollagen, as defined by Orekhovitch, is dissolved much more rapidly. The results obtained after silver staining are essentially the same as in the case of the Achilles tendon. Sodium periodate also dissolves long-spacing fibrils if allowed to act for some length of time.

In our electron miscroscope studies of this problem we have further been trying to answer the following three main questions:

(1) Are the parts in which silver is deposited actually identical with those in which phosphotungstic acid is deposited, i.e. the classical D-bands?

(2) Is periodic silver staining a general and reliable characteristic of mature fibrils, and irregular staining one of immature fibrils?

(3) Does such periodic silver staining actually take place within the fibril and throughout the depth of its cross section?

In order to be able to answer questions (1) and (2) it was necessary

to compare phosphotungstic acid staining with the silver staining after periodate action in various collagenous substances, among them substances that can be described as 'immature' collagen fibrils.

In several previous studies (Nemetschek, Grassmann and Hofmann, 1955) we have shown examples of highly subdivided fibrils taken from cats' tails and having 10 and more cross striations and of collagenous fibrils of cervical ligament in man. A highly subdivided fibril from a man's Achilles tendon is shown in Fig. 5. It was stained with phosphotungstic acid and reveals 13 cross striations. Fig. 1 shows the same substance after action of hyaluronidase and subsequently of periodate and silver. Periodic deposits with up to three bands per period can be observed. It is remarkable that the periodic silver deposits neither disappear nor diminish after treatment with hyaluronidase; on the contrary, we often find clearer pictures, since any deposits of carbohydrate-containing ground substance on the surface are removed.

The Achilles tendon of a human embryo (total length 14 inches) shows 10 intra-periods after having been treated with phosphotungstic acid. In the photographs so far obtained, however, there is too little contrast, and they are therefore not shown here. Weak, but typical periodic deposits could be obtained after silver staining was extended to 18 hours (Fig. 6).

It is not an established rule that the periodic silver deposits always occur in the dark bands of fibrils. Fig. 6*a* is a photograph stained with a combination of silver and phosphotungstic acid showing with sufficient clarity both the periodic silver deposits and the normal cross striation. Surprisingly enough we find an illustrative example of both possibilities: in one fibril the silver deposits can be clearly seen in the dark bands, in the other in the light bands.

Reticular fibrils from spleen are thin, show poor contrast and usually contain considerable amounts of interstitial substance. We have not yet heard of any photographs of highly subdivided cross striation. Fig. 7 shows a fibril with 10 intra-periods after treatment with phosphotungstic acid, whose intensity is slightly different from the normal. In the case of silver staining, the presence of considerable amounts of amorphous interstitial substance often proves disturbing, but such disturbances can largely be eliminated by previous treatment with trypsin or hyaluronidase. After such treatment we were able in a large number of cases to obtain periodic silver deposits in these materials also (Fig. 8).

In procollagen, highly subdivided cross striation has not been observed until recently (Kühn, Hofmann and Grassmann, 1956). The cross striation of procollagen is in part entirely identical with collagen, whereas certain intra-periods differ slightly in intensity. Fig. 9, for which phosphotungstic acid was used for staining, shows 13 bands per period. We were surprised, however, to find that all procollagen fibrils without any exception showed regular and very distinct intrafibrillar silver staining (Fig. 10). Three intra-periods can be discerned. If procollagen is a precursor of collagen, as asserted by Orekhovitch (Orekhovitch, Tustanowski and Plotnikova, 1948) it does not – in terms of the criteria of Schwarz and his team (Schwarz, 1955; Dettmer, 1955) – behave like immature but rather like mature collagen.

Stereoscopic photographs show that in periodic silver-stained fibrils a part of the silver grains is to be found on the surface, but another part lies apparently in the interior of the fibril.

In preparing long-spacing collagen from collagen solutions we have repeatedly observed fibril ramifications and starting from these sharply pointed fibrils projecting into the neighbouring area (Kühn, Hofmann and Grassmann, 1956). Such pictures give the impression that regular growth started from a nucleus, acting as a crystallization centre, into the neighbouring area. Could this nucleus inducing the formation of a fibril be an acid glycoprotein?

The silver staining of long-spacing fibrils, which has not been described so far, shows marked periodic deposits of silver grains. Up to 6 bands per period are discernible. Fig. 11 shows photographs taken of the same product, in one case not treated, in the other treated with periodate silver, and subsequently brought into register with each other. Such register can be clearly established because there are preparations in which the normal cross striation is evident through the silver deposits. A comparison reveals that the middle band, which is most distinct, both with and without phosphotungstic acid staining, by no means shows any outstanding affinity to silver. On the contrary, silver staining can also result in a marked silver impregnation of the two outer bands lying symmetrically on either side of the middle band. The electron-optical mass thickness obtained both with and without phosphotungstic acid is thus by no means always parallel to the silver deposits.

Anyone studying the formation of collagen fibrils from the amorphous carbohydrate-containing ground substance *in vivo*, or

the formation of specific collagen structures in the presence of certain polysaccharide fractions *in vitro*, or anyone studying in the electron microscope the dissolution of collagen under the action of periodate or the deposition of silver, will necessarily come to the conclusion that carbohydrate groups (or, to put it in a more cautious way, groups that react with periodate, thereby forming aldehydes) play an important part in the structure of collagenous fibrils.

But I should also like to draw attention to the somewhat difficult nature of this problem if we approach it from another angle, for example, by applying the classical methods for the determination of carbohydrates in proteins, such as the orcinol reaction, or the anthrone reaction which once helped us to establish the existence of carbohydrates in collagenous fibrils (Grassmann and Schleich, 1935).

TABLE I

CHANGE IN CARBOHYDRATE CONTENT OF CALF PROCOLLAGEN DURING RECRYSTALLIZATION

Sample	*Per cent Hexose*	*Per cent Hexosamine*
Procollagen impure	0.91	0.130
once recrystallized	0.84	0.085
twice recrystallized	0.73	0.011

When using these methods we found slightly less than 1 per cent hexoses and small varying amounts of glucosamine in collagen preparations. In previous experiments (Schneider, 1949) we tried to concentrate these carbohydrates in order to gain an insight into their composition and chemical linkage. At that time, this was done in accordance with the methods worked out by Rimington *et al.* (Rimington, 1931) for the examination of glycoproteins: partial hydrolysis with barium hydroxide, and subsequent precipitation with lead salts and mercuric salts. We were able to obtain products with a carbohydrate content of about 50 per cent, the yield being 30 per cent of the amount of hexose determined by means of orcinol in the starting material which is equivalent to roughly 0.3 per cent of the collagen. But by means of chromatographic and electrophoretic methods we later found (Grassmann and Kühn, unpublished) that

these products are not of a homogeneous nature and, even worse, that their composition differs according to the nature of the starting material employed. Electrophoresis established that these products can be divided into two fractions, one of which contains glucose, galactose, mannose and glucosamine in a molecular ratio of roughly 1:1:1:1, whereas the other fraction contains galactose and glucose 1:1, but is free of mannose and glucosamine. The starting material then was cattle corium which, apart from a brief treatment with calcium hydroxide had not been specially purified, therefore it cannot be said with certainty whether the small amounts of carbohydrates that were isolated in that way came from the collagen itself or from impurities in the starting substance.

The purification of the native collagen is a rather problematic affair, even though the material may, for example, be successively treated with salt solutions, calcium hydroxide and trypsin (Grassmann, Janicki and Schneider, 1937).

TABLE II

HEXOSE CONTENT OF SOME SAMPLES OF COLLAGEN AND PROCOLLAGEN
(Anthrone method)

Material	*Origin*	*Per cent Hexose*
Procollagen I rough	calf	0.91
Procollagen I once recrystallized	calf	0.84
Procollagen I twice recrystallized	calf	0.73
Procollagen II once recrystallized	cow	2.71
Procollagen III once recrystallized	cow	1.10
Procollagen III twice recrystallized	cow	0.98
Collagen pure*	cow	0.42

* Purification by extraction with NaCl, $Ca(OH)_2$ and treatment with trypsin (Grassmann, Janicki and Schneider, 1937).

Procollagen as defined by Orekhovitch, Tustanovsky and Plotnikova (1948) is much better suited for such studies since it can be purified by means of repeated reconstitution as fibrils. This method shows that glucosamine is not an essential constituent of collagen fibrils: repeated reconstitution eliminates it almost entirely. The hexoses, however, unlike glucosamine, decrease only slightly in

quantity (Grassmann and Wolf, unpublished). The hexose content of the collagen and procollagen preparations determined by the anthrone method varies slightly, depending on their origin and purification, but such variations are fairly small in the case of purified preparations. Various analytical methods give almost identical values for the hexose content of one certain preparation of twice recrystallized procollagen. The methods used are:

(1) Anthrone method.

(2) Hydrolysis with diluted acid, conversion of the amino acids into DNP-compounds and separation by means of ion exchangers.

(3) Spectrophotometrical determination as osazone after hydrolysis with diluted acid (Grassmann, Hörmann and Hafter, in press).

The results of these experiments show that the hexose content of a thoroughly purified procollagen amounts to roughly 0.7 per cent and it is not very likely that the standard methods, such as the anthrone method, etc., indicate a smaller quantity of carbohydrates than the material actually contains. We never found any higher amounts of carbohydrates in purified procollagen but unlike Moss (1955) we did not find any smaller quantities either.

Table III

HEXOSE CONTENT OF PROCOLLAGEN I (TWICE RECRYSTALLIZED) AND COLLAGEN ESTIMATED BY VARIOUS METHODS

Method	*Per cent Hexose* *Procollagen*	*Collagen*
(1) Anthrone, direct	0,73	0.42
(2) Hydrolysis (2 N HCl, 2 h, 110°), amino acids removed as DNP-derivatives on ion-exchangers, sugars determined with anthrone	0.69	0.40
(3) Hydrolysis (N HCl, 8 h, 100°), Osazone	0.90	0.50

It is doubtful if even this relatively small carbohydrate content is in true chemical linkage with the collagen. This was suggested by experiments to disintegrate them by means of trypsin. If heat-

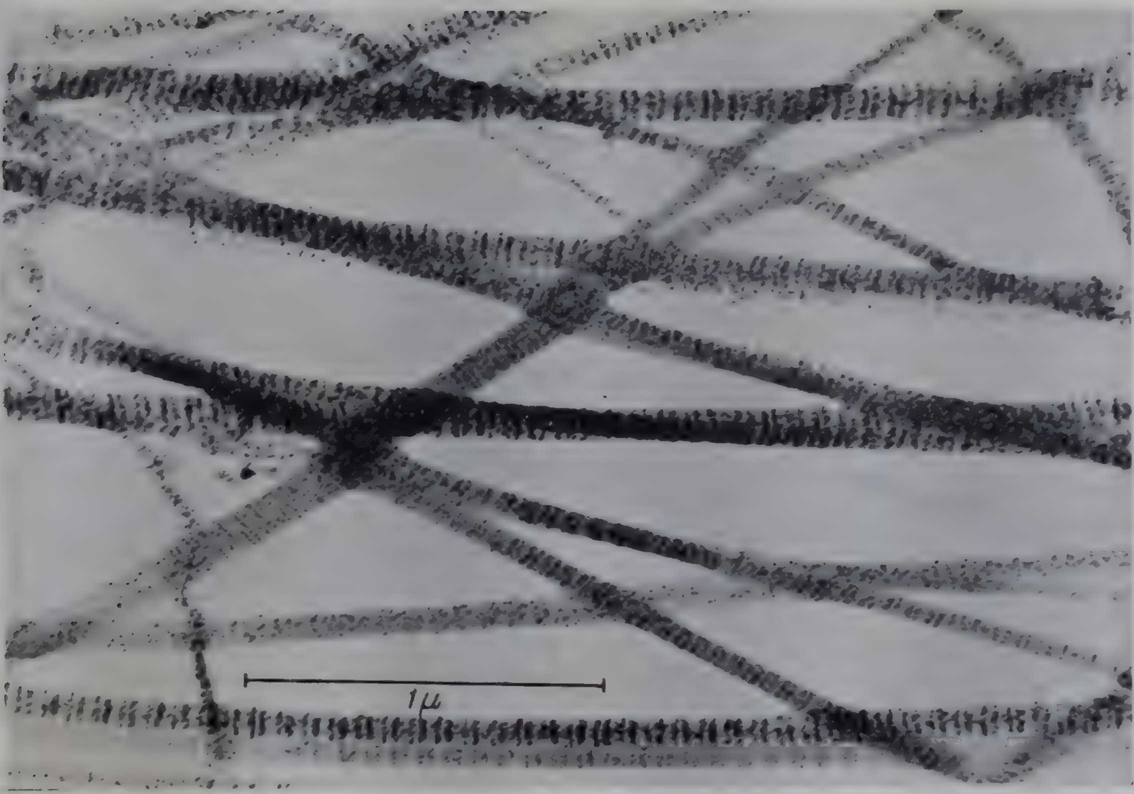


FIG. 1
Collagenous fibrils of human Achilles tendon treated with hyaluronidase, periodate-silver-urotropin-method (15 minutes' treatment with sodium periodate and 8 hours' silver staining). Electron microscope magnification × 12,900. Up to three bands per period visible.

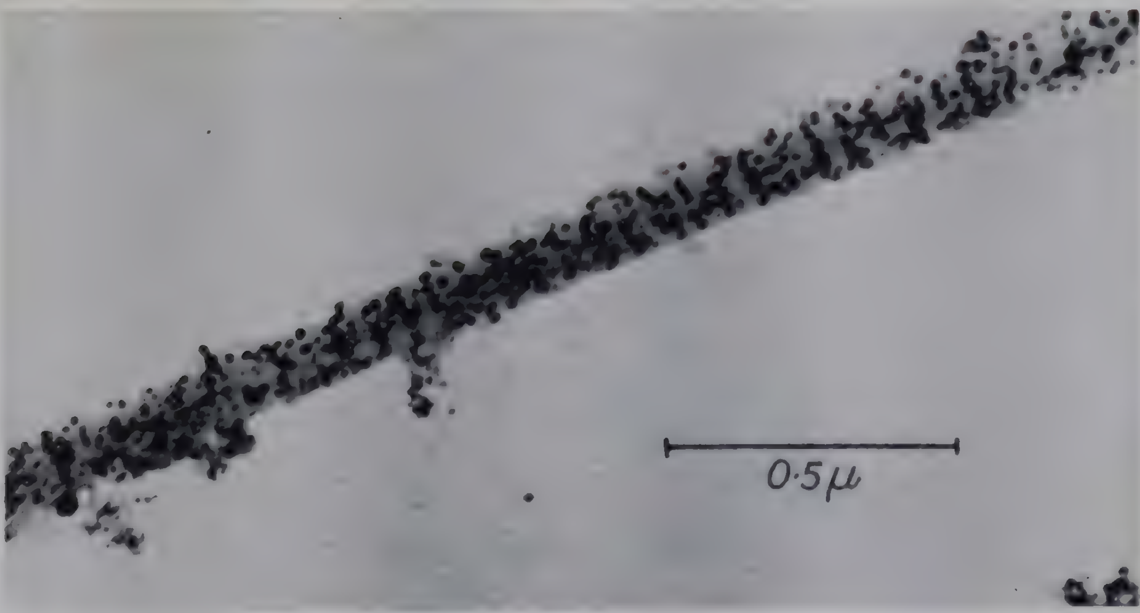


FIG. 2
Collagenous fibril of human Achilles tendon, after 1½ hours' treatment with $NaIO_4$ and 8 hours' silver staining. Electron microscope magnification × 12,900. The first fragments are splitting off from the fibril.

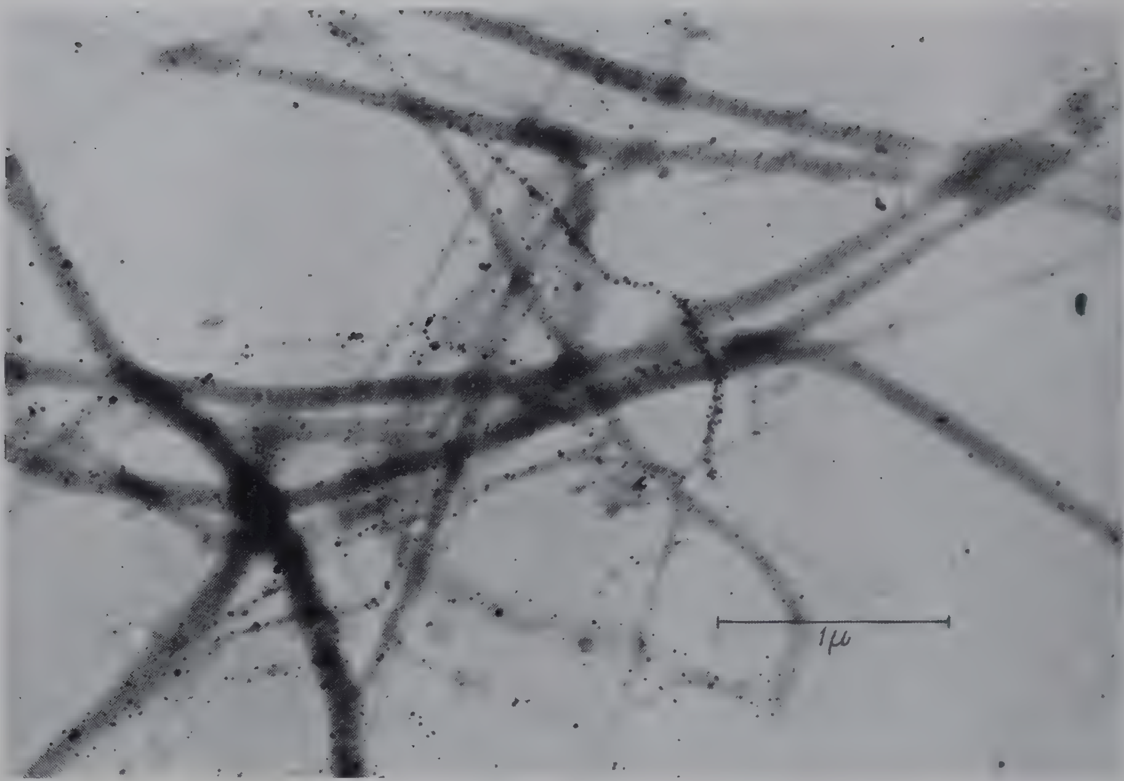


Fig. 3

Collagenous fibrils of human Achilles tendon after 18 hours' treatment with $NaIO_4$ and 8 hours' silver staining. Electron microscope magnification × 12,900. Only thin silver deposits. Cross striation is well recognizable.

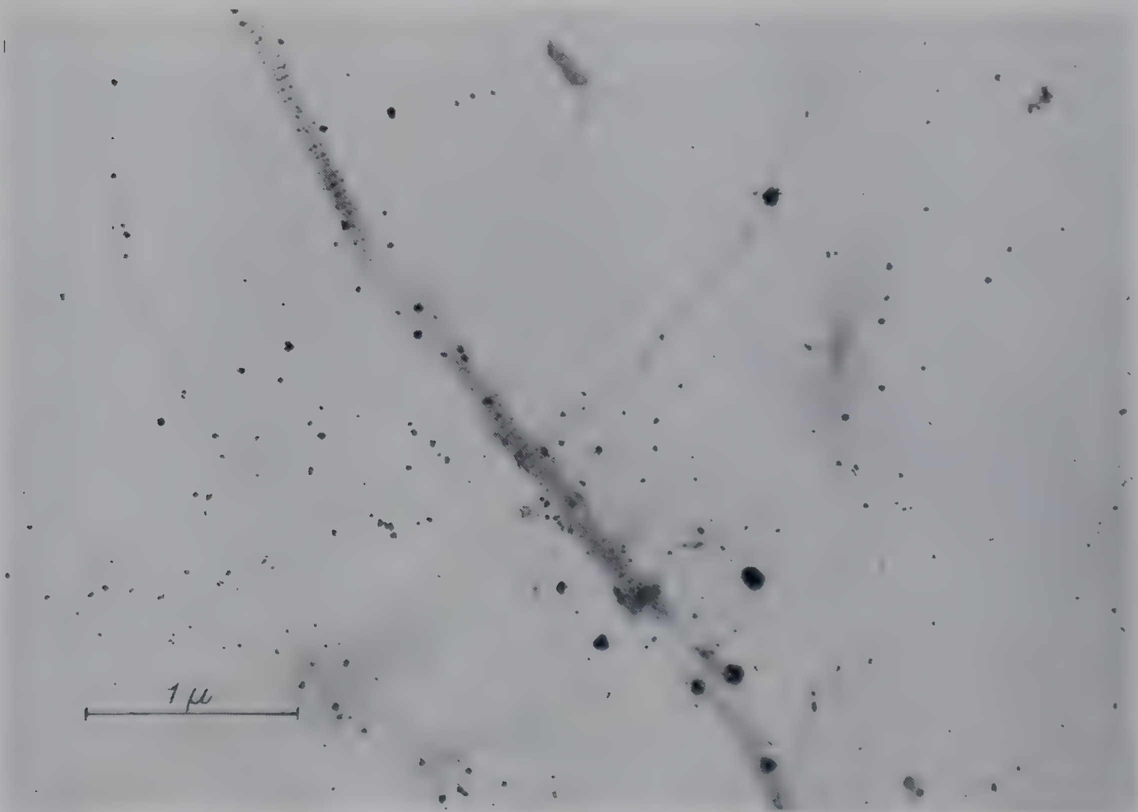


Fig. 4

Collagenous fibrils of human Achilles tendon, after 43 hours' treatment with $NaIO_4$ and 8 hours' silver staining. Electron microscope magnification × 12,900. Remaining fibril parts still reveal silver deposits.

FIG. 5

Collagenous fibril of human Achilles tendon, treated with trypsin and subsequently with phosphotungstic acid. Electron microscope magnification × 51,600

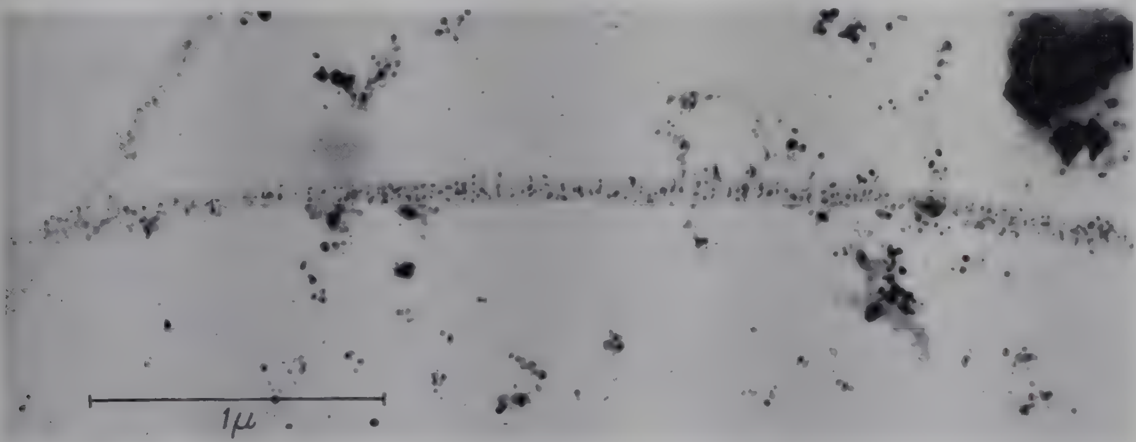


FIG. 6

Collagenous fibril of an Achilles tendon of human embryo, periodate-silver-urotropin-method (15 minutes' treatment with sodium periodate and 18 hours' silver staining). Electron microscope magnification × 12,900

FIG. 6a

Human Achilles tendon stained by silver-urotropin and phosphotungstic acid. Electron microscope magnification × 12,900. The silver deposits at I are situated in the light bands, at A in the dark bands of the cross striation.

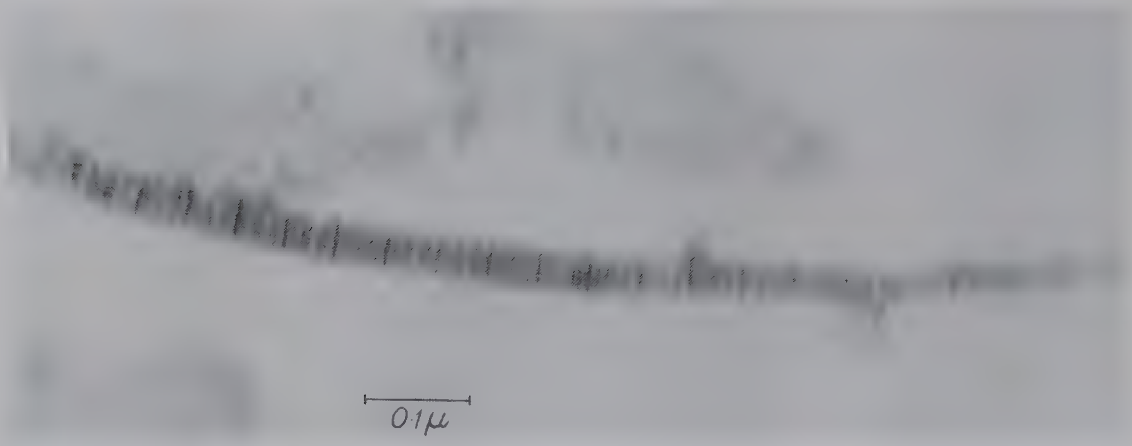


FIG. 7
Reticular fibril from cat's spleen, treated with hyaluronidase and subsequently with phosphotungstic acid. Electron microscope magnification × 51,600. 10 cross striations.

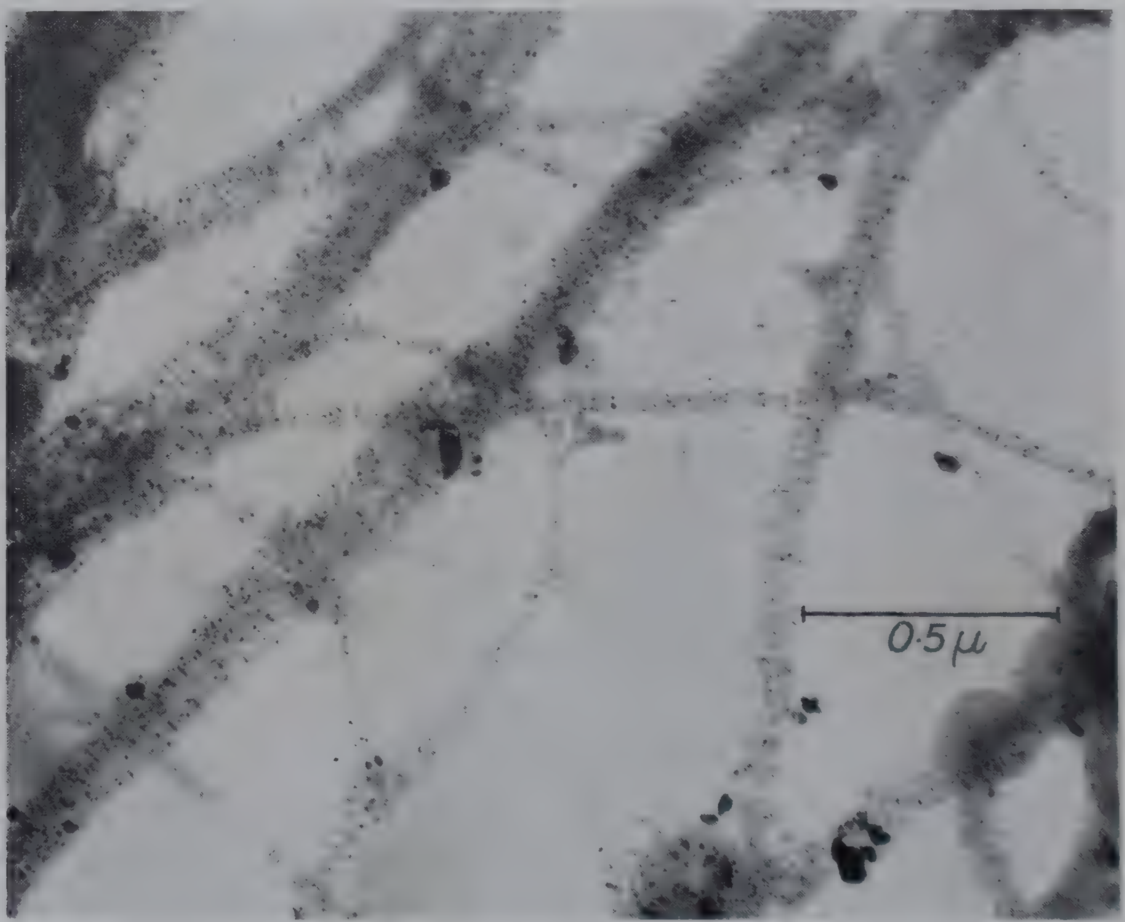


FIG. 8
Reticular fibril from cat's spleen treated with trypsin. Periodate-silver-urotropin method (15 minutes' treatment with $NaIO_4$ and 8 hours' silver staining). Electron microscope magnification × 12,900.

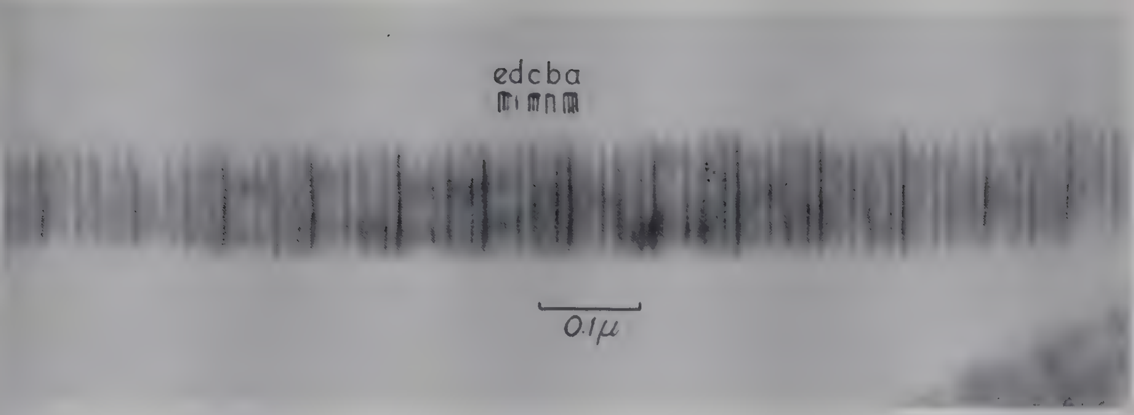


FIG. 9
Fibril of procollagen, treated with phosphotungstic acid. Electron microscope magnification × 51,600. 13 cross striations.

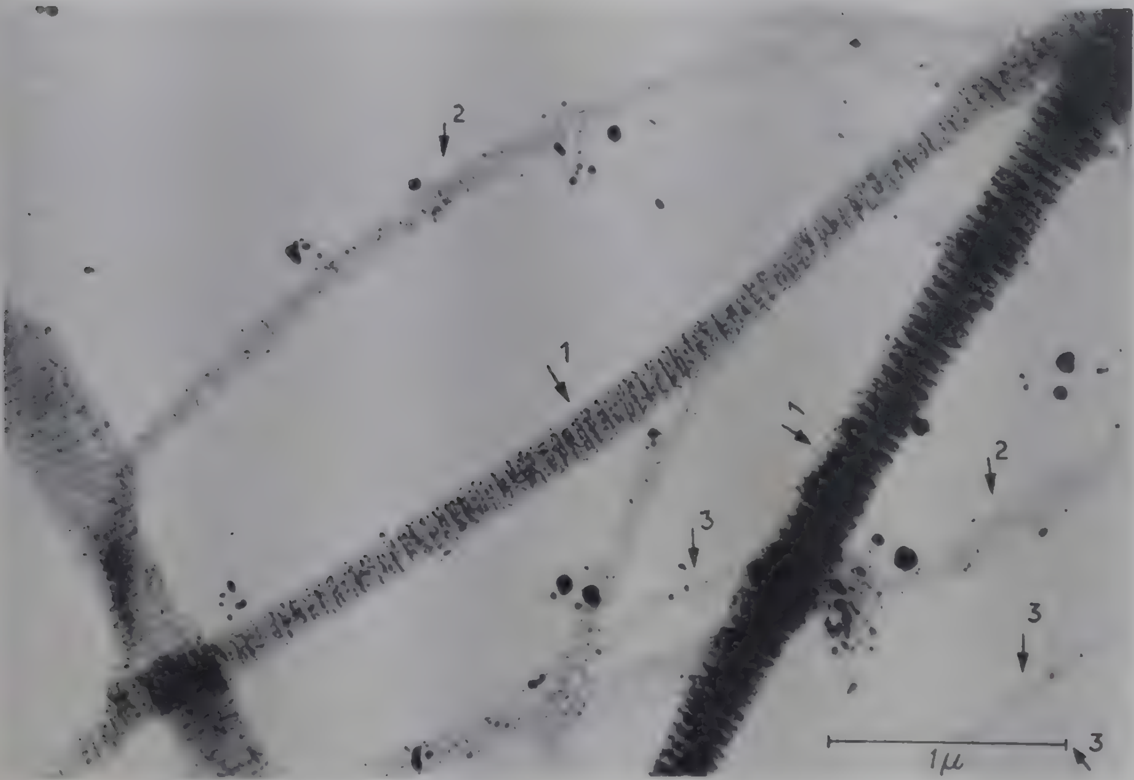


FIG. 10
Fibrils of procollagen, treated with trypsin, periodate-silver-urotropin method (15 minutes' treatment with sodium periodate, 8 hours' silver staining). Up to three silver cross striations per period visible.

Arrow 1: thick fibrils, very regular silver staining.

Arrow 2: Thinner striated fibrils, sometimes staining irregularly with silver.

Arrow 3: Thin fibrils, irregular silver staining.

Electron microscope magnification × 12,900.

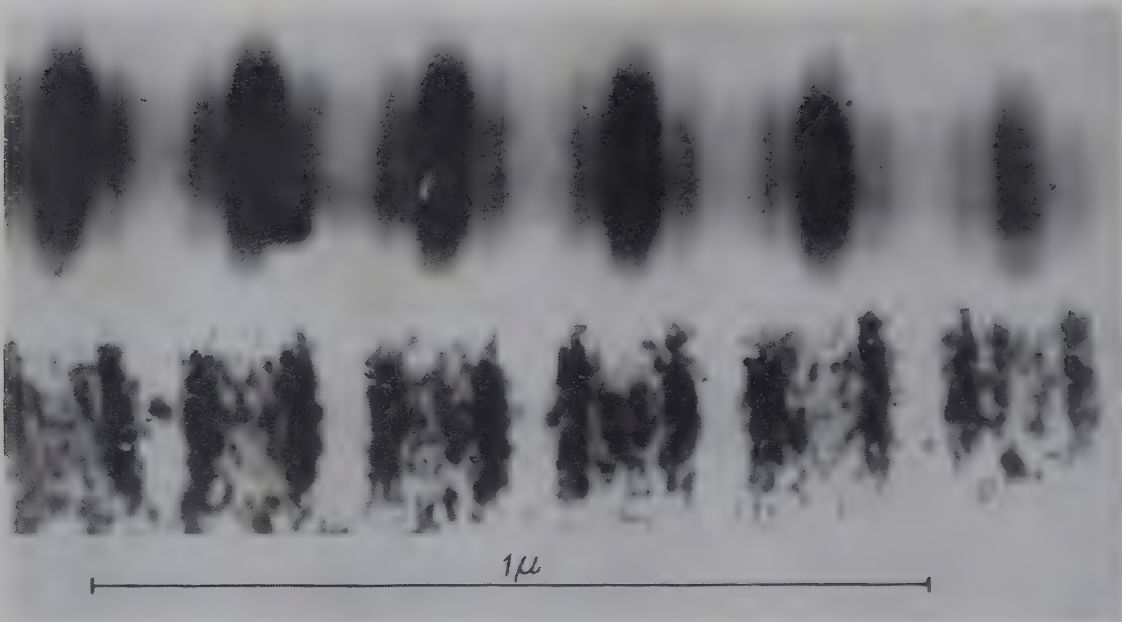


FIG. 11

Long-spacing fibrils prepared in an acidic solution of procollagen with α_1-glyco-protein treated with hyaluronidase. Electron microscope magnification × 12,900.

Top: without staining
Bottom: after treatment with periodate-silver.
Subsequently brought into register with each other.
Periods approx. 2000 Å.

treated collagen is treated with trypsin, a small amount (0.8 per cent) of a residue is left that is resistant to tryptic digestion. This residue contains more than 20 per cent carbohydrates (Grassmann, Hannig and Wolf, unpublished).

Considering such hexoses as may be determined by means of the customary standard methods it is obvious that hardly more than 0.5 per cent can ever have any true chemical linkage to protein. Such linkage could be possible in the following ways:

(1) N-glycosidic linkages between sugar and the amino groups of protein.

(2) O-glycosidic linkages to the hydroxy groups of hydroxy-amino acids.

(3) Ester linkages between the alcoholic hydroxy groups of sugars and the carboxyl groups of protein.

N-glycosidic linkages between sugars and amino acids are extremely unstable towards acids and bases (Micheel and Klemer, 1951, 1956), and N-glycosides form osazones with phenylhydrazine without any acid hydrolysis being necessary (Grassmann, Hörmann and Hafter, in press). The carbohydrates of collagen, however, form osazone only after having been split off by means of acid under such conditions as are required for the splitting of normal O-glycosidic linkages. A number of other findings, in particular also methylation experiments, indicate that part of the carbohydrate that is determinable by means of anthrone must be polysaccharides.

The existence of ester linkages in collagen and procollagen, on the other hand, must be considered highly probable because of the latter's behaviour towards lithium boronhydride (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished). According to all the previous findings, lithium boronhydride is not able to reduce free carboxyl groups in peptides and proteins whereas after esterification it reduces them easily to amino alcohols. It also does not split off peptide linkages (Hörmann, Grossmann, Wünsch and Preller, 1956). This makes it possible to determine the quantity of C-terminal amino acids in peptides and proteins. In the case of collagen and procollagen substantial amounts of amino alcohols of certain amino acids are obtained without previous esterification.

But are those small quantities of carbohydrates that can be traced by the anthrone and similar reactions really essential for collagen? Are they responsible for the dissolution and disintegration of collagen by means of periodate that were observed in our laboratory? I do not think so.

It has already been mentioned that, when splitting collagen or procollagen with periodate or phenyl iodosoacetate α-amino groups are set free whose quantity is equivalent to a medium chain length of 24 amino acids in the case of collagen and to 18 amino acids in the case of procollagen. The same chain lengths (Grassmann, Endres and Steber, 1954; Grassmann and Endres, unpublished), however, are found in the reductive disintegration of the presumed ester linkages by means of lithium boronhydride. But if there are really any 'weak joints' in the peptide chains of collagen that can be attacked by sodium periodate by oxidation and by lithium boronhydride by reduction, and if a disintegration of these 'weak joints' results in chain lengths of 24 or 18 amino acids respectively, it is obvious that, on the average, every 18 or 24 amino acids there must be such a 'weak joint'; and if these 'weak joints' were actually hexoses, at least 4-5 hexoses must exist per 100 mol of amino acids, which is equivalent to roughly 7 per cent by weight of carbohydrate. The hexoses that can be ascertained by means of the anthrone reaction are one-tenth of this amount.

This indicates that collagen and procollagen should contain a certain percentage of a carbohydrate-like substance of unknown quality. The amino acid analysis of collagen covers about only 94 per cent in weight, but nearly 100 per cent of the total N, as was found by Bowes, Elliott and Moss (1955) and can be confirmed by us (Grassmann, Hanning and Plöckl, 1955). The periodate consumption of procollagen which does not lead to any sharply defined endpoint, is substantially higher than it should be if the carbohydrate content were about 0.7 per cent (Grassmann, Hörmann and Fries, unpublished).

Are there any other known groups in collagen that may be responsible for such periodate consumption and for the dissolution of collagen and procollagen in sodium periodate?

There is no reason why the two hydroxy-amino acids, serine and threonine, should be affected by periodate, if they are part of a peptide chain. And, indeed, the quantity of these amino acids is hardly ever smaller after disintegration with periodate (Grassmann

and Kühn, 1955; Grassmann, Hörmann and Klenk, unpublished).

My colleague, Professor Zahn (Zahn, 1956), suggested that hydroxylysine, present in collagen in a quantity of 0.7 per cent, also in peptide linkage, might fulfil all the requirements for an action of sodium periodate; it must split off formaldehyde if disintegrated. It is true that formaldehyde is formed if sodium periodate acts on procollagen, and its amount is in conformity with the amount of hydroxylysine present (Grassmann, Hörmann and Fries, unpublished). But the periodate consumption is far higher than would be justified by the sum of hydroxylysine and such carbohydrate as can be determined by anthrone, quite apart from the fact that an oxidation of the side chains of hydroxylysine would hardly account for a disintegration of collagen and procollagen by sodium periodate, and would certainly not explain the formation of α-amino groups.

Compounds of such kind as are formed from N-glycosides by means of the Amadori rearrangement (Amadori, 1925) have recently been isolated from biological sources.

FORMULA 2

They do not react with anthrone (Borsook, Abrams and Lowy, 1955) and we also found that they produce osazone (Grassmann, Hörmann and Hafter, in press) under the action of phenyl hydrazine without any previous acid hydrolysis being required. It is therefore highly improbable that such anthrone-negative carbohydrate groups are the cause of the reaction with periodate.

I have considered it my task to show you how many questions still cannot be answered in respect of the groupings of a carbohydrate nature in collagen. Formerly, the main difficulty and the crux of the

problem seemed to be that the carbohydrate content of the collagen fibril was small, and that it was difficult to decide whether such carbohydrate content was part of collagen itself or due to any impurities of the amorphous ground substances. At present I am inclined to see the crucial point of the problem in the fact that the groups responsible for the histological and electron microscope silver staining and the disintegration by periodate are not identical with the ones that can be traced by means of the hitherto customary carbohydrate reactions. It will be necessary, and we are at present trying, to explore these still unknown groupings in the disintegration products of collagen.

GROUP DISCUSSION

Dr. Reed inquired about the exact conditions of Dr. Grassmann's experiments with periodate as regards pH, temperature, etc. Dr. Grassmann replied that 4 per cent periodate was used at pH values of 7 to 8 and phenyl-iodoso-acetate at pH 3.4 and the temperature was 40° C. Controls were done without periodate.

Dr. D. S. Jackson stated that in his experiments on the effects of periodate on tendon, solution of the collagen depended entirely on the presence of swelling. If approximately 2M sodium chloride were included in the reaction mixture, then the fibres could be left almost indefinitely without any dissolution taking place at all. This suggested that whatever the periodate was reacting with, it might be a cross-linkage rather than along the backbone chain. Thus the effect of periodate may be to reduce the stability of the fibres which then give a lower shrinkage temperature which would be of the order of the temperature of the experiments of Dr. Grassmann. Hence the fibres would first shrink and then form a type of gelatin.

Dr. Grassmann recalled that in his electron micrographs collagen fibrils still retained good cross banding even after treatment with periodate.

Dr. D. S. Jackson asked how long in Dr. Grassmann's experiments was periodate allowed to react before complete dissolution of the collagen was obtained.

Dr. Grassmann replied that for procollagen 60 per cent was dissolved within half a day, but collagen took longer.

Dr. Meyer recalled that periodate was originally introduced into carbohydrate chemistry splitting glycol groups with the production of aldehyde, but this was done under certain defined conditions. In an unknown excess of periodic acid, such as used in the experiments described by Dr. Grassmann, there was an indiscriminate splitting of C-C bonds which had no relation to this glycol structure. He wondered whether one could draw any conclusions as to the action of the periodate under the experimental conditions described. Could Dr. Grassmann detect with his methods the disappearance of some amino acid under the action of periodate? Did he use the Moore and Stein method?

Dr. Grassmann said no, but they did use electrophoretic separation into groups which proved very accurate except in the case of hydroxyproline. There was no disappearance of amino acids under the action of periodate.

Dr. Neuberger felt very doubtful about the application of periodic acid in the way Dr. Grassmann had suggested and he wished to support Dr. Meyer. It was quite clear that a cis-glycol or an alpha-amino beta-hydroxy-amino acid is split very quickly by periodic acid, but he felt that peptide linkages might be broken after long exposure to a large excess of

periodate. Could there not be a very small amount of destruction? Another point was the presence of small amounts of tyrosine (no one had ever obtained a collagen free from tyrosine), the molecules of which should be attacked by periodate.

Dr. Gross said Dr. Grassmann was ascribing the silvering process to the 0.5 per cent sugar in the collagen fibrils. However, collagen contained a little over 1 per cent hydroxylysine in which the epsilon amino group is probably free. This being so, the hydroxylysine is most likely to be oxidized as readily as the sugar to yield aldehyde groups to react with silver salts.

Dr. Grassmann said it is indeed so that hydroxylysine is the only amino acid which fits the conditions for attack by periodate. When bound in the peptide chain serine and threonine should not be attacked within the limitations stated by Dr. Meyer. In his own experiments, formaldehyde was set free in a quantity exactly equivalent to the hydroxylysine content. Thus, hydroxylysine is certainly attacked. But why should the peptide link break and an alpha amino group result if anything happens to the side chain of hydroxylysine?

Dr. Partridge asked if Dr. Grassmann had considered the possibility of the oxidation of methionine by periodic acid? It was present in collagen to the extent of 0.8 to 0.9 per cent which represented about six residues per 100,000 grammes. If it were oxidized it would produce locally situated sulphur containing compounds (which can catalyse photographic processes at least) and the small number of such residues could explain the cross bands described by Dr. Grassmann.

Dr. Gustavson mentioned some work he had done on the effect of pretreatment of calf-skin collagen (limed) in solutions of sodium periodate on its reactivity, which had some bearing upon the mechanism of the periodate reaction on collagen. The pretreatment, at 20° C. and pH 4.5-5.0, lasted for 5-7 days; solutions of various concentration of periodate being employed. $NaIO_4$-solutions less than 0.25 molar had no appreciable solubilizing effect, neither did they lower the shrinkage temperature of collagen and its co-ordination capacity (hydrogen bonding), for vegetable tannins for instance. However, the binding of cationic chromium complexes was somewhat lowered, indicating impaired reactivity of the carboxyl groups. The function of the cationic protein groups was unaltered. Since periodate solutions of 0.25 molar strength are able to oxidize carbohydrates during the experimental conditions applied, it appears that the small amount of sugar present in thoroughly limed collagen does not contribute to its stabilization. Solutions of greater strength, such as 0.5 molar $NaIO_4$, have a drastic effect on collagen. About 80-90 per cent of the bovine skin collagen is solubilized. The shrinkage temperature is lowered from 60-65° C. to 35-40° C. Moreo

the binding capacity of collagen for co-ordination compounds, reacting by hydrogen bonding on the keto-imide group of collagen (mimosa tannins), is more than doubled. Also increased reactivity of the treated collagen by means of its anionic and cationic groups is observed. The periodate appears to rupture the stabilizing cross-links of the protein, making new hydrogen bonding loci available for reaction (on the –CO.NH– linkages mainly). However, the cleavage of some keto-imide bonds of the backbone is indicated to occur. According to Grassmann and Kühn the amino acid composition of collagen is practically unaffected by the periodate treatment. Sodium perchlorate shows exceedingly strong lyotropic effects on collagen also, as evident by the destruction of collagen, impaired thermal stability, and drastically increased co-ordinate reactivity. These effects occur very suddenly on raising the concentration of the perchlorate solution above one molar. It looks like the periodate-collagen reaction is very complicated, involving far-going alterations of the protein units, apart from the effect on the carbohydrate component which reaction probably is of minor importance.

A FEW REMARKS ON CONNECTIVE TISSUES RICH IN MUCOID

A. van den Hooff

(1) Among the several types of connective tissue existing, that of the cornea shows some remarkable features. The most important are: complete transparency, regular structure and lack of blood vessels.

Histologically the cornea is characterized by its metachromatic stainability with toluidine blue. The cornea shares this property with some other types of connective tissue: aortic wall, umbilical cord and cartilage, also tissues without vascularization.

Firstly a few remarks will be made on these tissues with special reference to the cornea.

Metachromasia may be seen as proof of the occurrence of certain mucopolysaccharides; histologists apply the term 'mucoid' to indicate a mucus-like substance impregnating the tissue.

The four tissues mentioned (cornea, cartilage, umbilical cord and aortic wall) are characterized at the electron microscope level by the occurrence of thin collagenous fibrils. The diameters are 250-300 Å in cornea and aortic wall, 300-400 Å in umbilical cord; 70 per cent of the cartilage fibrils are thinner than 300 Å. Mucoid may be visible as amorphous material enveloping the individual fibrils. The amount of visible mucoid is increased very much in the case of atheromatous degeneration of the aortic intima.

Mucoid is easily washed off; in replicas of dried material (prepared without washing) its presence is indicated by the lack of the typical collagen cross-banding (compare Figs. 1 and 2).

The isolated thin fibrils have a cross-banding of 210 Å in shadowed preparations (Fig. 3). They are indistinguishable from those in mesenchyme of embryonic skin and from the fibrils of reticulin.

The coincidence of metachromasia and small fibril diameter is striking; this is the more conspicuous since the presence of mucoid in the cornea is secondary, i.e. the deposition occurs in the last part of embryonic development. This could be observed during the

development of chick embryos. Up to the twelfth day there is a decrease in metachromasia both in cornea and sclera; the twelfth and thirteenth day metachromasia is practically absent. From the fourteenth day onward metachromasia in the cornea increased gradually, on the last – twenty-first – day of embryonic life the maximum intensity is reached. The sclera stains orthochromatically from the twelfth day onwards. The cornea becomes transparent at about the same time: from the fourteenth to the twenty-first day the amount of transmitted light increases from 60 to 100 per cent.

Smits has made hexosamine determinations in corneas and scleras of bovine foetuses of increasing age. Up to a total length of 25 cm. both tissues show a decrease in hexosamine; from 25 cm. onward the hexosamine content of the cornea increases again, while the content of the sclera decreases further. After birth the hexosamine-HCl content of cornea and sclera are 2.48 per cent and 0.58 per cent respectively.

(2) Among non-histologists there is a tendency to call thin collagenous fibrils 'reticulin'. As reticulin is defined as connective tissue fibrils showing argyrophilia, it is not justifiable to identify the aforementioned fibrils with reticulin, because the mucoid-containing tissues cannot be impregnated with silver. So fibril thinness in itself is not sufficient to cause argyrophilia; therefore thin fibrils, which can be impregnated with silver (reticulin) must be distinguished from thin fibrils lacking this characteristic.

Fibrils of both types were examined with the electron microscope after impregnation with silver. Care was taken that possible relations between fibrils and interfibrillar substance were maintained to a certain extent, therefore a simple mechanical fragmentation technique was applied. Cornea and cartilage were chosen as tissues rich in mucoid, spleen and kidney as organs containing much reticulin. Embryonic skins of increasing age were studied also, as in developing skin there is a transition of reticulin into collagen.

The results can be summarized as follows: whenever argyrophilia is present – recognizable at the electron microscope level by big silver grains – a membrane-like matrix is visible between the fibrils (Fig. 5); this applies to the reticulin of spleen and kidney; in young embryonic skin the matrix can be shown also, but the membranous character is less obvious. The fibrils of cornea and cartilage are covered with very small silver grains – corresponding with the

brown colour in the light-microscope (Fig. 4); the presence of a matrix as mentioned above could not be shown. Apparently the matrix of reticulin has a far greater mechanical stability than mucoid which can be washed off rather easily.

Instructive are the pictures one can observe in the same specimen of spleen of a new-born pig (Fig. 6): the reticulin fibrils – recognizable by the big grains – show clearly the presence of cementing substance (Fig. 6*a*); Fig. 6*b* shows fibrils which are non-reticulin, as shown by the bundling of the fibrils and the smallness of the silver grains; a matrix as seen in Fig. 6*a* is lacking.

These results give support to the view that argyrophilia bears no relation to fibril diameter as such, but rather to a specific bond between fibrils and matrix.

GROUP DISCUSSION

Dr. Gross stated that Dr. Marie Jakus at the Retina-Foundation in Boston had demonstrated long-spacing collagen fibrils in cornea similar to those precipitated by glyco-protein, and having a 1000 to 2000 Ångström spacing. In Descemet's membrane, which by X-ray diffraction and chemical analysis consists primarily of collagen, Dr. Jakus has shown a beautiful regular three-dimensional network with spacings of an hexagonal type, 1000 to 1500 Ångström apart. Cornea is the only known tissue to date that normally has long-spacing fibrils.

Dr. Fitton Jackson inquired whether Dr. van den Hooff had examined embryonic tissue earlier than ten days. Dr. van den Hooff said yes, at seven days, but it was impossible to measure transparency in the material earlier than eight days. Dr. Fitton Jackson said that by using polarized light, laminated layers of fibres can be seen in ten- or eleven-day-old embryos.

Dr. Neuberger asked how the constituents of the cornea compared with those of the sclera in terms of wet weight.

Dr. van den Hooff replied that he had no wet weight figures but the dry weight cornea contained about 50 per cent collagen, 5 per cent mucopolysaccharide and 30 per cent non-collagenous protein. Sclera (dry weight) contained 80 per cent collagen, 1 per cent mucopolysaccharide and about 10 per cent non-collagenous protein.

Dr. Meyer said this problem of transparency of the cornea was an intriguing one. Maurice tried to explain it on the basis of a diffraction grating formed by the different fibril layers. On drying the transparency disappeared and it became translucent. In this respect, hyaline cartilage,

which has a much higher carbohydrate content, is translucent in thin layers, but not transparent. If one uses hydraulic pressure on the cornea it is possible to extract a great deal of polysaccharide of two different types — one with electrophoretic mobility of a non-sulphated polysaccharide and the other of a sulphated polysaccharide. There is metachromasia of the substantia propria, but Descemet's membrane shows no metachromasia. The question is this: is Descemet's membrane collagen or not? Dr. Gross said yes; it contains 10 per cent hydroxyproline and twice that amount of glycine.

Dr. Meyer said the grating theory did not entirely explain the opacity of the cornea. It must have something to do with alteration in the hydration of the tissue components. It would also be altered if a new type of collagen grew in, as in leucoma.

Dr. van den Hooff agreed that the transparency of the cornea was only within very narrow limits of hydration; even pressure on the cornea would render it opaque. Apparently the changes in refractive index of the fibrils by tension are sufficient to make the cornea opaque.

Dr. Fitton Jackson said that presumably the pressure closed up the 'lattice'. The refractive index of normal cornea is 1.52 and when this is altered by hydration or dehydration then the optical properties of the cornea impair sight owing to a change in the refraction index between the fibres and interfibrillar materials.

Dr. Snellman mentioned work done some years ago by Engstrom and Caspersson which showed that the different layers of the cornea had different refractive indices.

Dr. Gillman pointed out that the lens seemed to have received much less attention than the cornea. In certain metabolic disturbances the opacity of the two may run together. He wondered, in particular, whether any studies were available at the electron microscope level, on extracted or sectioned material, on alterations in the morphology of the fibres in the cornea or the lens in experimental diabetes. In alloxan diabetes development of corneal and lens opacity seem to be closely related to carbohydrate metabolism. The other point was that as in the arteries, so too in the lens and in the cornea, alterations in the metabolism of the individual could produce tendencies towards the binding of certain substances, for example in the arteries, i.e. lipids and minerals. Thus calcium, iron and lipid may be more easily bound on corneal fibrils in the presence of particular types of systemic metabolic disturbances affecting corneal metabolism.

Dr. Meyer said he did not know of any correlation between corneal and lens carbohydrate moieties in both the cornea and the lens opacity apart from infections as in vitamin deficiency states. He did not know that the two went hand in hand in diabetes.

Dr. Gillman said they can, under certain experimental conditions. Opacity of the cornea is not always a function of infection. For example, in riboflavine deficiency, vascularization of the cornea may supervene and lead to opacity unassociated with infection.

Dr. Meyer presumed that this was due to the production of abnormal collagen fibrils during the vascularization process.

Dr. Gillman said his main point was that the morphology and chemistry of the fibres of the lens and cornea did not seem to have received the attention they merited either by the light or the electron microscope.

Dr. Schwarz pointed out that the first function of the cornea was transparency. A change in fibril or a change in cementing substance would alter the refraction. The second function was the maintenance of a permanent curvature for the whole life of the organism. Astigmatism can be corrected surgically by an incision at the corneal-scleral junction which alters this curvature.

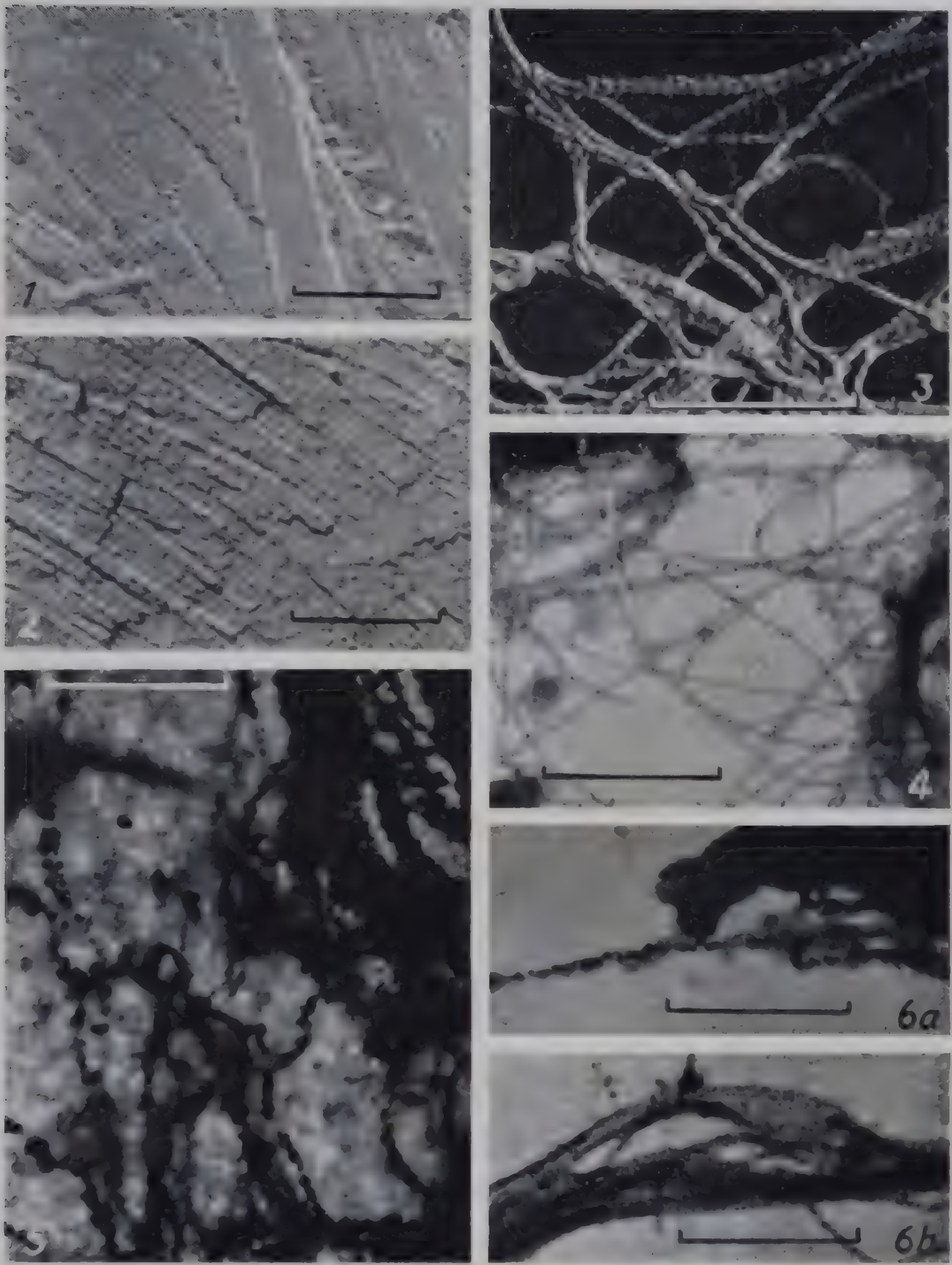


FIG. 1
Replica of bull's sclera. × 15,000

FIG. 2
Replica of bull's cornea. × 15,000

FIG. 3
Fibrils of bull's cornea. × 20,000

FIG. 4
Fibrils of bull's cornea, impregnated with silver. × 17,000

FIG. 5
Reticulin from kidney of bovine foetus of 60 cm., impregnated with silver. × 17,000

FIG. 6
Fibrillar material from the spleen of a new-born pig: the pictures are of the same specimen. (*a*) reticulin, (*b*) collagenous material. × 17,000

WHAT IS RETICULIN?

A. H. T. Robb-Smith

Reticulin fibres were first described by Kupffer in 1876 and eight years later Mall (1888) claimed that it was distinct from collagen; since then its nature has been a recurring matter of dispute, in the late 'twenties from a histological viewpoint, and in the last few years in the light of advances in molecular biology.

As a biological structure of microscopical dimensions, reticulin can only be defined in terms of its morphological and staining characters, as revealed by the light microscope. It would seem desirable to relate it, if possible, to the various collagens which are characterized by differing chemical, physical or structural features.

The generally accepted definition of reticulin is that it consists of fine (1μ) isotropic fibres which show true branching, usually at right angles; it stains faintly with acid fuchsin (van Gieson's stain), accepts the acid aniline dyes in the Mallory or Masson Trichrome method, and appears black in both toned and untoned sections impregnated with silver by the Maresch-Bielchowsky method.

There has been some disagreement as to its reaction with the periodic Schiff reagent, according to the particular technique used, but the majority of observers agree that reticulin stains strongly, whereas collagen fibres stain faintly or not at all (Robb-Smith, 1952); with the sulphation metachromasia reaction of Kramer and Windrum (1953-54) reticulin is metachromatic, while collagen is orthochromatic.

Native collagen and reticulin fibres resist tryptic digestion but undergo dissolution when incubated with clostridial collagenases (Robb-Smith, 1945, 1953; Dresner and Schubert, 1955).

In the identification of reticulin it is not sufficient to depend on one character alone, and there is no doubt that in the strict sense in which it is proposed to use the term here it would be quite wrong to assume that all fibres which appear black in the light microscope when impregnated with silver by one of the modifications of the Maresch-Bielchowsky method are reticulin. These silver impregnation methods are not histochemical tests and for them to be meaningful, it is necessary for both the solutions and material to be controlled

critically as it is perfectly easy for any of the tissue elements in a preparation to appear black, brown or yellow if the conditions are so adjusted.

In mature connective tissue, reticulin often appears to merge with collagen fibres but the angular branching is very characteristic and predominantly it is seen in relation to the basement membranes between connective tissue and epithelium and around muscle and nerve fibres, etc.

However, it has been shown (Lillie, 1952) that the staining reactions of basement membranes are not uniform, suggesting variability in their constituents and although in general it would be correct to say that reticulin is a component of mammalian basement membranes, there are exceptions to this. For example, the basement membrane of the normal human renal glomerules contains no reticulin and its staining reactions are markedly different (Rinehart *et al.*, 1953), while electron microscopic studies (Benedetti and Tivibelli (1954), Pease (1955) have failed to reveal the presence of collagen-type fibrils.

This is of some importance as Cruickshank and Hill (1953), using the fluorescent antibody technique of Coons and Kaplan (1950) showed that anti-glomerulus serum 'stained' both renal glomerular basement membrane and reticulin, but not collagen fibres and they quite rightly stated that their experiments showed the presence of a common antigen in these two tissue elements, but this work has been misinterpreted as a specific method for identifying reticulin.

In embryonic and regenerating connective tissue, the angular branching reticulin fibres are seen quite early (in the human foetus by 38 days, in healing wounds in 6-8 days) but another type of argyrophil fibre is also present which has often been called reticulin, but I believe should be distinguished from it; there are fine wavy fibres which do not branch and merge very closely with the developing collagen fibres and indeed appear to be replaced by the collagen fibres. I think these should be regarded as immature, argyrophil, if you like, collagen fibres, and clearly if they are to be called reticulin then a different name must be adopted for the angular branching argyrophil fibres of basement membranes and mature connective tissue.

There is a further variety of argyrophil fibres which has been very little studied and the only site in which I am confident of their existence is the ovarian stroma (Robb-Smith, 1952). They are pre-

sent in association with reticulin and I have only been able to display them by enzymic digestion as they are collagenase resistant but are trypsin soluble; after collagenase treatment they are orthochromatic with sulphation metachromasia, but no significance can be attached to this, as one has no idea what other changes have been induced in the tissues by this technique.

Bembridge (1952) has described collagenase-resistant trypsin digestible fibres in the ciliary region of the vitreous and Martin (1953) observed with the electron microscope fine unbanded trypsin soluble fibrils in cartilage, but it is impossible to say whether or not they are of a similar nature, although this serves to emphasize that one cannot assume that an argyrophil fibre is a reticulin fibre.

Since the days of Nageotte and Huzella, it has been shown that precipated collagen can be rendered black with silver impregnation methods, but these fibres formed *in vitro* should not be designated reticulin if the term is to be used for the biological structural element which I have already described. Experiments along these lines may well be of value in elucidating the factors that determine the argyrophil reaction, yet because of the relative non-specificity of these reactions under abnormal conditions, I do not believe attempts should be made to draw very close analogies between results obtained in this way and the structure and nature of reticulin as I have defined it, and for the same reasons, I do think too much significance can be attached to the interesting experiments of Irving and Tomlin (1954).

In my department we have been interested in reticulin for a good long time and in an endeavour to characterize it more precisely, it was necessary to find a tissue rich in reticulin and poor in collagen, and after a survey of a wide range of tissues, we adopted the subcortical tissue of the kidney as its fibrillary portion consisted almost exclusively of reticulin, as I have defined it here, and it was comparatively easy to dissect out the small amounts of collagen around the blood vessels.

Using material of this type, Kramer and Little (1952) studied it with the electron microscope and showed that it consisted of a feltwork of fine (down to 100 Å) randomly arranged fibrils, having the 640 Å periodicity and more recent work on sectioned material has confirmed these findings. X-ray diffraction photographs (Little and Windrum, 1954) showed a pattern quite characteristic of collagen, but in addition to the collagen bands other rings were seen at

2.5, 2.35 and 2.2 Å. This material dissolves very slowly on heating with 5N HCL and leaves a large quantity of brown residue but is readily soluble in boiling normal sodium hydroxide. Windrum, Kent and Eastoe (1955) have investigated the chemical nature of this material. It consists of 85 per cent protein and its amino acid composition is very similar to that of collagen. Carbohydrate is present to the extent of 4.2 per cent non-hexosamine, the sugars identified being galactose, mannose and fucose; no uronic acid or sulphate ester was detected. In addition, it contained 10.9 per cent bound fatty acids of which about 95 per cent was myristic acid, the rest palmitic. Thus, it appears that reticulin as I have defined it, from human kidney, is a lipo-glyco-protein in which the amino acid constituents are very similar to those in collagen.

It seemed desirable to try and ascertain what relationship, if any, reticulin had to the various soluble collagens that have been described; any isolation process appeared unsatisfactory as it would be difficult to relate the isolated fragments with histologically defined structures, and so an attempt was made to expose tissues to various buffer solutions and then examine them histologically.

The tissue used was human kidney, obtained six hours after death from a child, who had died as a result of an accident and showed no pathological lesions in the viscera. Portions of kidney, unfixed, were cut on a freezing microtome and slices about 100µ thick were obtained. Some of these were immediately fixed in 4 per cent saline formaldehyde as controls (Figs. 1 and 2), others were placed in large volumes of the buffer solutions and gently agitated while being kept in a refrigerator at approximately 2° C.; at the end of the period of exposure, they were fixed in 4 per cent saline formaldehyde. All the tissues were embedded in paraffin and sections were cut at 7µ and stained by the usual methods for connective tissue – haematoxylin and eosin, van Gieson, orcein, toluidine blue, sulphation metachromasia of Kramer and Windrum, Masson's trichrome, periodic acid Schiff and Robb-Smith's silver impregnation method (1937). For each staining method all the sections were treated simultaneously for identical times in order to avoid uncontrolled variations in staining.

The solutions to which the unfixed sections of kidney tissue were exposed were as follows:

(1) 0.2 M phosphate buffer pH 8.8 for 12 hours; (2) 0-1 N acetic pH 2.73 for 12 hours; (3) 0.005 N sodium hydroxide pH 12

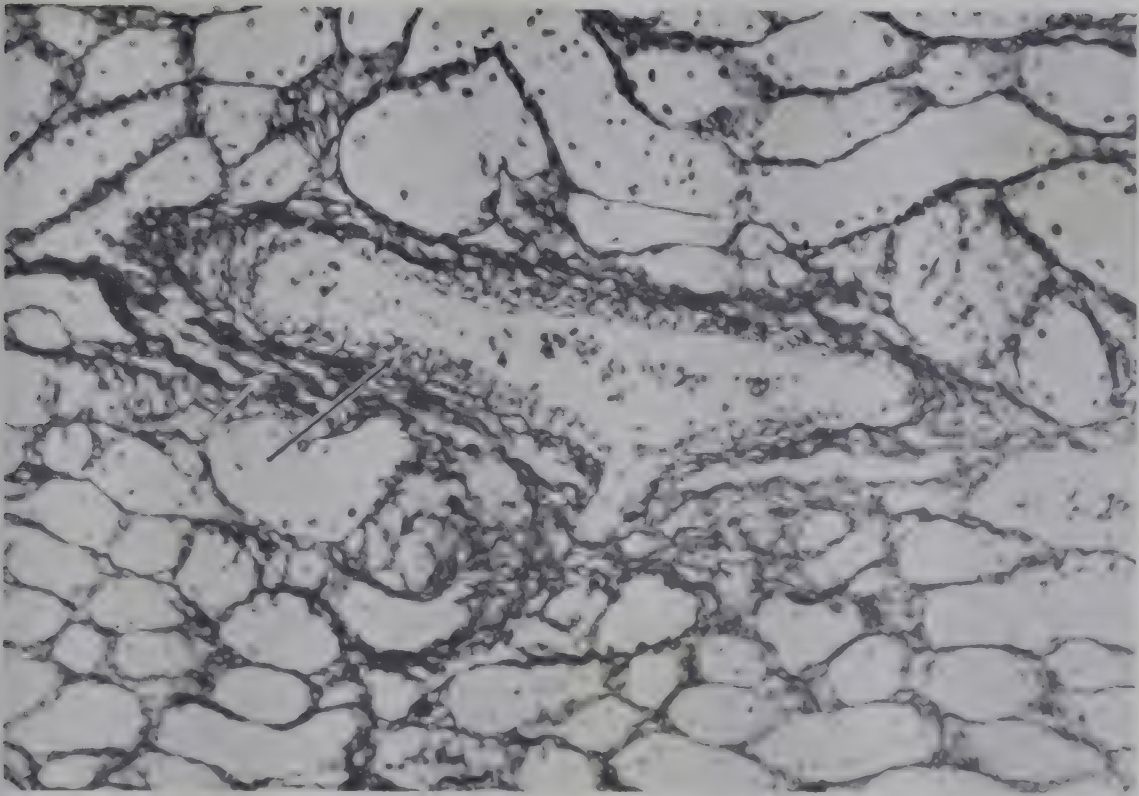


FIG. 1

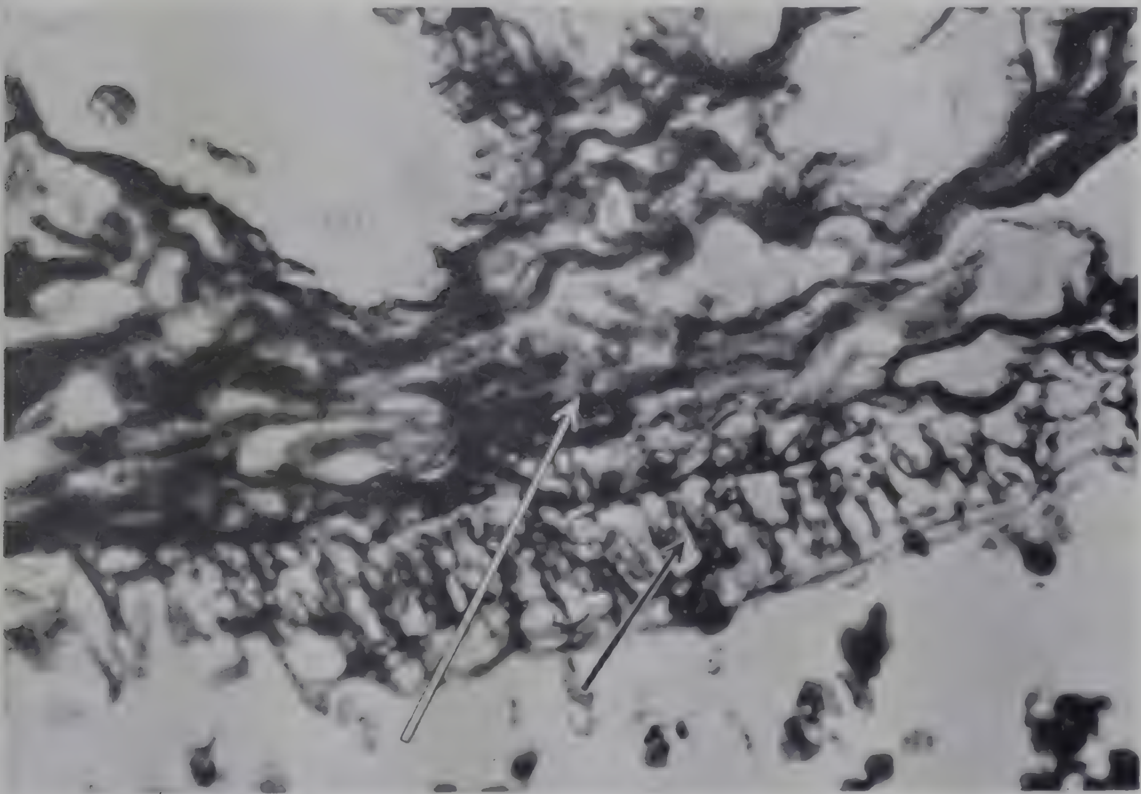


FIG. 2

A. Control material fixed in formalin without prior exposure to buffers, Fig. 1 × 420; Fig. 2 × 1,400.

Human kidney exposed to acids and alkalies and then fixed in formalin: paraffin sections were prepared and stained with silver for reticulin fibres.
The arrows indicate the sites of reticulin and collagen and it will be noted that there is no change in the reticulin stain, although there is obvious diminution in the staining of the collagen and ground substance.

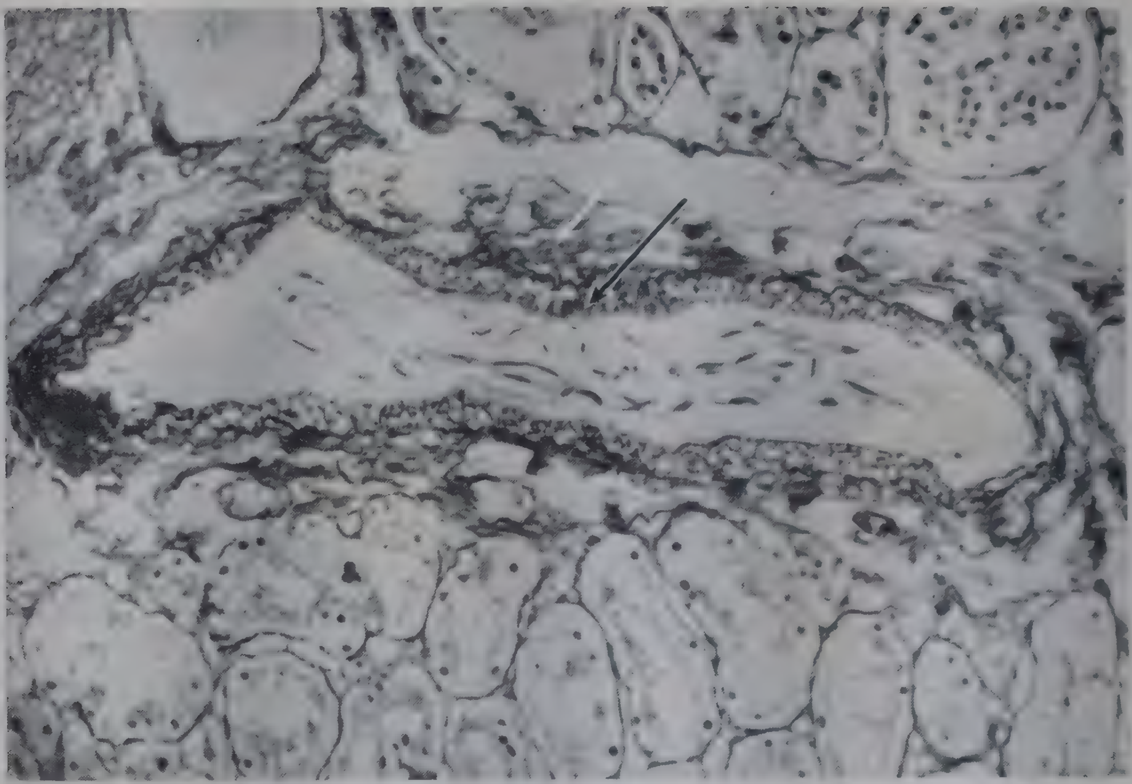


Fig. 3

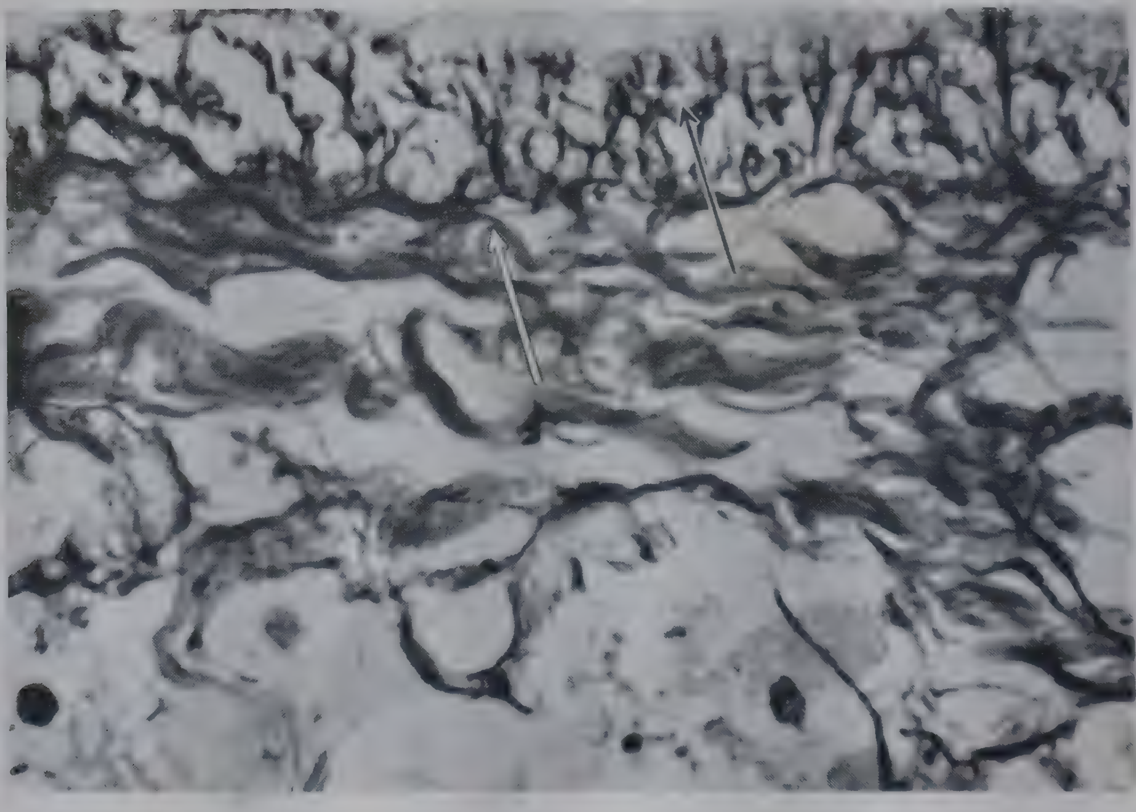


Fig. 4

Fig. 3. Phosphate buffer pH 8.8 for 12 hours at 2° C. followed by 0.1 M citrate buffer pH 3.8 for 12 hours at 2° C.
Fig. 3 × 420; Fig. 4 × 1,400.

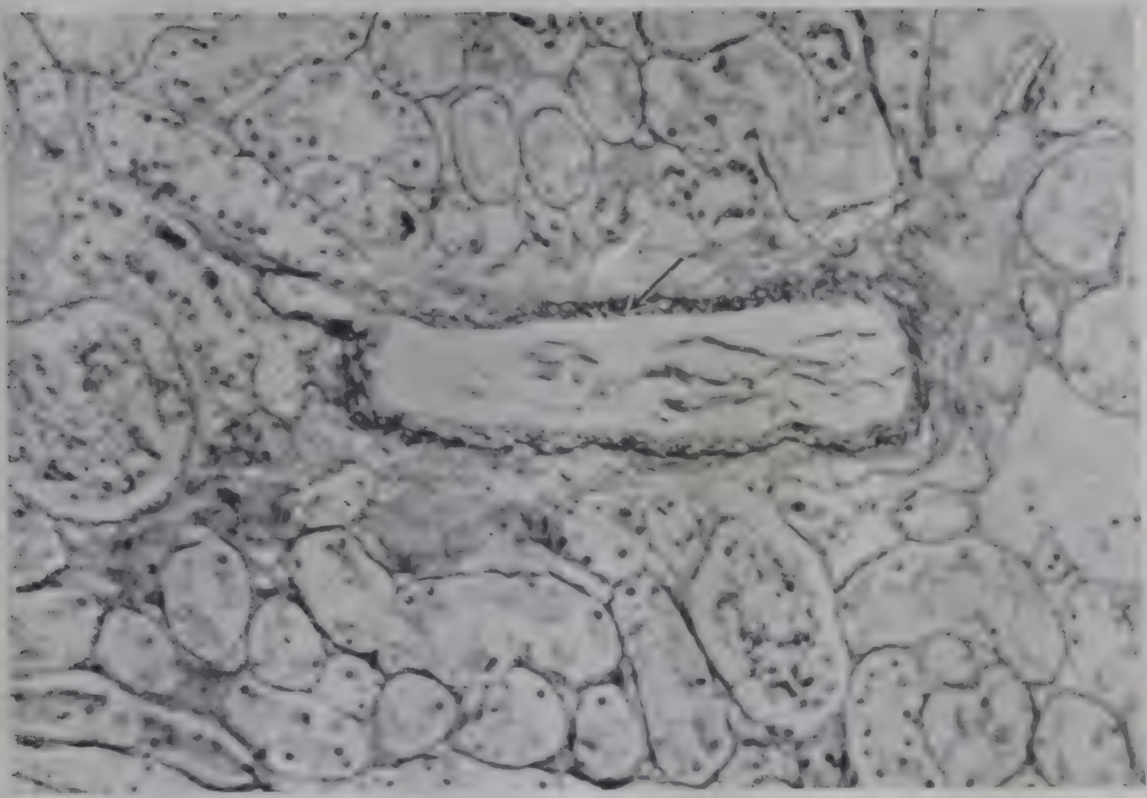


FIG. 5

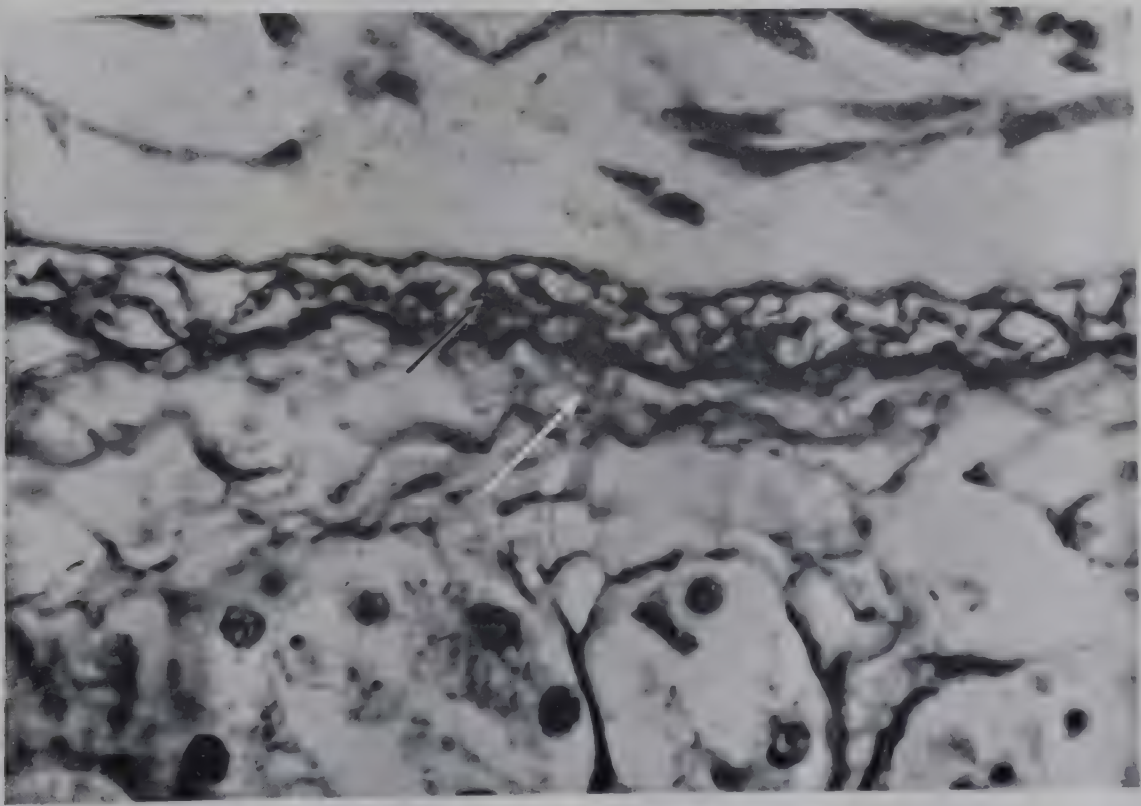


FIG. 6

C. Phosphate buffer pH 8.8 for 12 hours at 2° C. followed by acetic acid pH 2.7 for 12 hours at 2° C.
Fig. 5 × 420; Fig. 6 × 1,400.

for 12 hours; (4) 0.1 M citrate buffer pH 3.8 for 12 hours (Figs. 3 and 4); (5) phosphate buffer for 12 hours followed by acetic acid for 12 hours (Figs. 5 and 6); (6) phosphate buffer 12 hours, followed by alkali for 12 hours; (7) phosphate buffer for 12 hours followed by citrate buffer for 12 hours.

It is not proposed to describe in detail the histological changes that have been observed, but in essence it was found that following the exposure of the tissue to their various buffers, there was no tinctorial or structural alteration of the reticulin as observed with the light microscope but that there were marked alterations in the staining reaction of the collagen and ground substance in all the preparations, except those exposed to phosphate buffer alone. The most striking diminution of collagen staining was observed in the sections treated with phosphate buffer followed by citrate buffer and phosphate buffer followed by acetic acid.

A similar experiment was set up in which the material used was granulation tissue from a healing wound which was rich in the fine wavy non-branching argyrophil fibres which I have mentioned on page 178. In these experiments it was found that the buffer solutions had no effect on argyrophil reticulin fibres, but there was disappearance and loss of silver staining of the wavy argyrophil fibres and again this was most marked in the sections treated with phosphate and citrate buffers and phosphate buffer and acetic acid.

The methods of extraction used correspond closely to those adopted for the extraction of soluble collagens and although no attempt was made to demonstrate a soluble collagen in the buffers after the tissue had been in them, yet the histological evidence, for what it was worth, suggested that collagen had been extracted from the tissue and also that there had been extraction of immature argyrophil collagen fibres, but no change could be detected with regard to reticulin.

There have been few, if any, integrated morphological and chemical studies of connective tissue after extraction of chemically identifiable fractions, but a relevant study is that of Tustanowski *et al.* (1954) on collastromine (there may well have been more recent papers on this subject which have not been read). Tustanowski *et al.* showed that after repeated extraction of rat skin with citrate buffer pH 4.0 and final extraction with water at 50° C. for an hour and a half, the residue consisted of fibres which were argyrophil, but did

not show the periodicity of collagen with the electron microscope, the material was examined 'histochemically' – the methods used were staining with picrofucsin, Foot's silver method, toluidine blue and periodate fuchsin – and the results were interpreted as indicating that the fibres consisted of an albumen-mucopolysaccharide complex with an argyrophilic fibrillar albumen and this they called 'collastromine'. They could identify collastromine in the residue which had not been heated, but felt that in these samples there was admixture with 'procollagen' and suggested that collagen should be interpreted as a many phase system whose main components are collastromine and procollagen.

The Russian workers do not discuss the possibility of their residual fibres being reticulin as interpreted here, but we have shown that samples of reticulin as prepared by the method of Kramer and Little (1953), on boiling, lose the collagen periodicity, as shown by the electron microscope, although hydroxyproline can be identified in both the insoluble residue and the supernatant, but carbohydrate can only be detected in hydrolysates of the insoluble residue.

However, X-ray diffraction studies may suggest a difference between collastromine and reticulin. Tustanowski *et al.* say that in the residue after citrate extraction, the interference maxima at 2.9 Å and 11.4 Å disappear only leaving diffuse rings at 4.5 Å and 9.8 Å and this persists on heating; Little and Windrum (1954) on the other hand, stated that X-ray diffraction photographs of reticulin show the pattern characteristic of collagen together with bands at 4.5, 3.8, 2.5, 2.35 and 2.2 Å, which they interpret as confirming the presence of saturated fatty acids, principally myristic acid that had been identified chemically by Windrum, Kent and Eastoe (1956); furthermore, Little and Kramer (1952) mention that in boiled reticulin the collagen pattern is lost, but there is an intense ring at 4.5 and 3.8 and there are also inner rings of very low intensity which are difficult to measure accurately.

It can be seen that the main discrepancy between collastromine and reticulin lies in the statement by Tustanowski that the unheated residue still contains some 'procollagen' but does not show the X-ray diffraction pattern of collagen or the collagen periodicity on electron microscopy, whereas our group found that unheated reticulin did appear to consist in part of a collagen-like material. Furthermore, it must be recognized that the residue which the Russian workers studied almost certainly contained a number of other connective

tissue components such as elastica, and detailed chemical analysis of the residues after extraction of collagen from connective tissue have not, as yet, been made; nevertheless, it seems very probable that reticulin accounts for some of the properties of collastromine.

In this paper, I have tried to show the difficulties in identifying reticulin and the progress that has been made up to date. Reticulin as defined here does not appear to be a precursor of the mature collagen fibre, but a biological fibre of the collagen family, of considerable stability, which is unlikely to form the structural basis of the collagen fibre. It is desirable to distinguish reticulin as defined here from other argyrophil fibres which in the past have been called 'reticulin' of which the most important example is the immature argyrophil collagen fibre to be found in areas of fibrillogenesis whether embryonic or reparative.

GROUP DISCUSSION

Dr. Snellman said the silver staining might be due to aldehyde groups derived from any acetal.

Dr. D. S. Jackson said that the major proportion of the fatty acid is myristic, and palmitic and stearic might be impurities.

Dr. Robb-Smith replied that at first he also thought it was an impurity but, as it was found in constant amounts, he was forced to the conclusion that it was not.

Dr. Consden stated that the work of Eastoe showed conclusively that myristic acid was released only after hydrolysis. Combination of myristic acid with protein might account partially for the resistance of reticulin to different treatments. Dr. Consden was interested in the autoclavibility of reticulin in order to remove it from tissue residues. He had found, using kidney reticulin, that about 85 per cent of hydroxyproline could be extracted after autoclaving for nine hours. However, a small amount continued to come out if autoclaving was prolonged.

Dr. Meyer said sialic acid was a strong reducing agent and easily split off. It occurred in granulocytes, peptides and carbohydrates.

Dr. Reed asked Dr. Robb-Smith whether there was an increase of elastic tissue staining after treatment with phosphate buffer at pH 8.8. Dr. Robb-Smith replied that there was no increase in elastic staining with orcein as far as the blood vessels were concerned; but the tissue had been exposed to the buffers for only 12 hours at 2° C.

Dr. Partridge asked Dr. Consden whether his residue after autoclaving had been examined for polysaccharide. Dr. Consden replied that un-

fortunately 50 per cent of inorganic material was present so it could not be analysed accurately. It contained very little hydroxyproline after continuous autoclaving but did give a positive carbohydrate test.

Dr. Schwarz inquired whether reticulin maintained its stage of differentiation. Dr. Robb-Smith said of course, it could be damaged by a number of processes and disappear, but once it was laid down as basement membrane it probably would not change to normal mature collagen. Reticulin was not a half-way house to mature collagen fibres but a 'branch line'.

SOME NEW ASPECTS OF THE STABILITY AND REACTIVITY OF COLLAGENS

K. H. GUSTAVSON

INTRODUCTION

It should be noted at the outset, that my contribution will be restricted firstly to a review of results, partly unpublished, which have been obtained on investigating the nature of the valency forces responsible for the stabilization of the polypeptide chains present in the protofibrils and the filaments of collagen. Thus, reactions and forces involving distances less than 50–100 Ångström units are concerned (Bear, 1952). Information on the type of side chains and links in the polypeptide backbone forming the sites for these valency forces will hence be a principal issue. A comprehensive review has recently been given by Gustavson (1956a).

Secondly, some problems, which might be involved in the alteration of collagen upon ageing and under pathological conditions, will be briefly surveyed, particularly the possible role of the setting up of high concentration gradients of electrolytes in the interface on collagen in two-phase systems, and the implications of such enrichment of the solute at the solid phase on the properties of collagen. Attention will also be drawn to the possibility of the occurrence of cross-linking and tanning processes as an explanation of the alteration of collagen in the organism under certain conditions.

TYPE OF CROSS-LINKS

It is generally considered that the cohesive forces between the elementary units of the collagen fibril are of three types:

(1) *ionic valence*, mainly in the form of salt-like cross-link between oppositely charged, long side chains, as those between the glutamic acid and lysine residues, the commonly cited example (Speakman and Hirst, 1931, 1932; Lloyd *et al.*, 1933).

(2) *non-ionic* cross-links, the most important one being the hydrogen-bond, e.g. the short link formed by the keto-imide groups on adjacent protein chains (Pauling and Niemann, 1939). The presence

of other intermolecular hydrogen bonds, such as the link formed by the hydroxy group of the hydroxyproline (= hypro) and the keto-oxygen of an adjacent keto-imide group has been indicated experimentally also (Gustavson, 1953, 1954a, b; 1955a).

(3) Van der Waals forces, and similar links formed by other weakly polar groups.

To which extent these various intermolecular forces, or types of co-ordinate bonds, contribute to the stabilization of the fibre structure is the second cardinal question. Using the degree of the hydrothermal stability of collagen, i.e. the value of the temperature of the instantaneous shrinkage of the fibre in water, the *Ts*, some indication, or an approximation of the relative importance of the ionic and the non-ionic protein groups in the cross-linking of collagen is obtainable (Gustavson, 1942a, b; 1946a).

It appeared to the present author that by ascertaining the effect of the complete inactivation of the ionic groups of collagen, the acid and base binding ones, on the shrinkage temperature by an agent which is irreversibly fixed by collagen and which does not swell the protein, such as the naphthalene sulphonic acids (Bergmann *et al.*, 1930), an approximation of the degree of stabilization of collagen effected by the ionic linkage should be feasible. The blocking agent to be used for the severance of the salt-like bridges must be irreversibly fixed by collagen and must not swell it, since by swelling of the collagen secondary alterations in the hydrogen bond type of cross-links take place, which will further complicate an already intricate problem. By saturation of collagen with β-naphthalene sulphonic acid on equilibration with solutions of pH 1.5, the shrinkage temperature of calf-skin collagen is lowered 12-14° C. (Gustavson, 1942). This impaired stability is generally conceived to be the result of eliminating the ionic forces between the polypeptide chains. Similar results have been obtained by Jackson (1953, 1954) on tendon collagen. By the treatment of the skin in concentrated solutions of some strongly lyotropic agent, such as calcium thiocyanate of 2-molar strength, the fibre will shrink in the treatment, even at temperatures of 10° C. or lower (Küntzel, 1937). There are indications favouring the view that the lyotropic shrinkage is intimately connected with the breaking of some of the numerous cross-links of the hydrogen bond type in collagen (those on the —CO.NH— groups primarily) (Gustavson, 1942a). The large *Ts* decrease (△ *Ts*) noted is to be ascribed to the impairment of all types of intermole-

cular stabilizing bridges. It is of the order of some 60° C., whereas the degree of stabilization by the salt-like links is of the order of 12-14° C. on the *Ts* scale only. Thus, such a large amount as at least 75-80 per cent of the total sum of the internal cohesive forces in the collagen lattice has to be assigned to the non-ionic cross-links. The purely electrostatic function of the salt-like cross-link should be expected to be rather slight in view of the effect of the great D.E. constant of the aqueous solutions on the fibres.

At this point it is appropriate to note that the effective valency range of the sites of the ionic link is of the order of 50-100 Ångström units, while that of the dipolar forces responsible for the hydrogen bond and for the co-ordinate bonds generally is a few Ångström units only (Bear, 1952). Then, the latter forces cannot be operative between larger units of the collagen (skin) structure, such as fibres, whereas electrovalent links may be able to extend their force across interfibrillar spaces of some 50-60 Ångström units. They may accordingly influence the mechanical strength of the fibres, their cohesion. It is noteworthy that in the light of the available experimental material, the *hydrothermal stability* of collagen appears to be governed by the short links between polypeptide chains (helices), and thus to be a function of the number and the strength of the hydrogen bonds present (Gustavson, 1948), whereas the *tensile strength* of a *single fibre bundle* apparently is mainly determined by surface forces of weakly polar nature, influencing the frictional forces between the fibres. This view receives some support by the fact that by cross-linking of collagen by means of such small molecules as formaldehyde, the shrinkage temperature is very markedly increased, while the tensile strength is not materially affected (Highberger, 1947; Gustavson, 1948; Jacobson and Lollar, 1951; Mao and Roddy, 1950; Roddy, 1952).

TYPE OF HYDROGEN BONDS

As to the number of hydrogen bonds of the ordinary type on the –CO.NH– groups, generally conceived to be the principal ones (5) – and also believed by some to be the only type existing – it appears that presently one systematic set of hydrogen bonds is assigned to each unit of three residues of the trihelical configuration of collagen by Rich and Crick (1955) and by Crick (1956). The most recent model consists of three polypeptide chains, each having a

three-fold screw axis with the chain winding round each other to form a coiled-coil (Crick, 1956; Cowen *et al.*, 1955; Ramachandran, 1956; Ramachandran and Kartha, 1954, 1955; Reed *et al.*, 1956). The sequence: gly-pro-hypro can be accommodated in this model (Kroner *et al.*, 1953; Schroeder *et al.*, 1954). In view of this recent development, according to which the molecule of collagen consists of a unit of three helices, it should be noted that the term *intermolecular* or *interchain* cross-link, as used in earlier publications as well as in the present paper, should be identified with the link connecting two molecules of the trihelical unit type, while the *intramolecular* cross-link should apply to bonds between the three polypeptide chains in the unit.

The presence of strong hydrogen bonds between the hydroxy group of the hypro residue and some other link or group on an adjacent polypeptide chain, probably the keto-oxygen of the keto-imide group, in the first place, has been indicated by the results from chemical investigations (Gustavson, 1954a, b; 1955a). The data from X-ray diffraction studies show that an intermolecular bond $-OH \ldots OC\langle^{NH-}$ can readily be accommodated in the trihelical model, and actually that such a cross-link is a logical consequence of this particular structure. This type of link was independently suggested by Huggins (1954) in his latest model of collagen, based on data from X-ray diffraction, and by the present author (1954a, b) from his investigations of the chemical reactivity and the hydrothermal stability of various types of collagen.

HYDROTHERMAL STABILITY AND HYDROXYPROLINE CONTENT

In studies of the hydrothermal stability of collagen of skins of mammals and fishes (mainly bovine skin and skin of cod fish, respectively), measured by the temperature of instantaneous shrinkage, *Ts*, it was found that the *Ts* of native skins of mammals generally falls in the range 60-70° C., while those of fish skin are much lower, for skins of cold-water fish 35-45° and for skins of warm-water fish 45-55° C. (Gustavson, 1953; Takahashi and Yokoyama, 1954). It was pointed out in the early papers (Gustavson, 1942b, c; 1949; 1950b) that the hydrogen bond type of cross-link appeared to be less developed in the collagen of fish skin than in that of bovine skin. Since information on the amino acid composition of these collagens,

particularly that of fish-skin collagen, was too fragmentary at that time, no definite explanation of the greater availability of non-compensated keto-imide groups indicated in the teleostean collagen was possible. The complete analytical data of the amino acid content of collagen of bovine skin (Bowes and Kenten, 1948) and fish skins, which were later forthcoming (Neuman, 1949; Neuman and Logan, 1950), showed the main difference between collagen of mammals and teleosts to be the low content of hypro (9 per cent) of fish-skin collagen compared to 14 per cent for bovine collagen, apart from

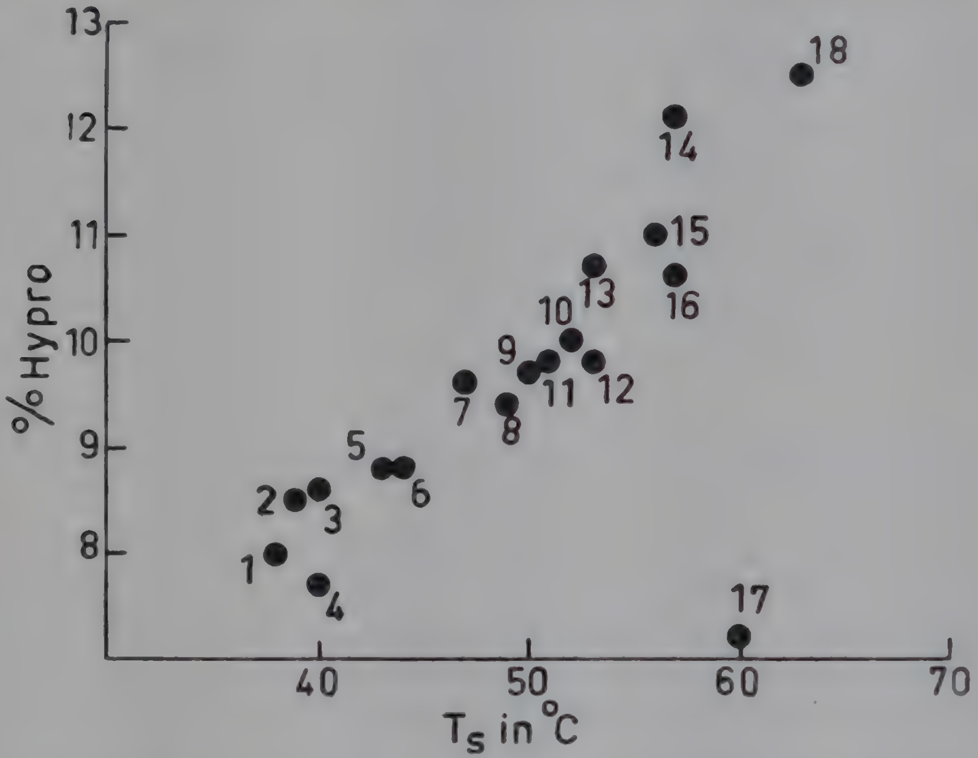


FIG. 1

The relation between the Ts (in ° C.) and the hydroxyproline content of collagens of the skin of various fishes in the native state

The numbers on the dots represent:

(1) Alaska pollak, *Theragra*
(2) Atka mackerel, *Pleurogrammus*
(3) Flathead flounder, *Hippoglossoides*
(4) Codfish, *Gadus*
(5) Flounder, *Tanakius*
(6) Small-mouthed sole, *Limanda*
(7) A kind of flounder, *Lepidosetta*
(8) Japanese halibut, *Paralichthys*
(9) Japanese mackerel, *Scomber*
(10) Yellowtail, *Seriola*
(11) Jack mackerel, *Trachurus*
(12) Fresh water eel, *Anguilla*
(13) Great blue shark, *Prionace*
(14) Wild goldfish, *Carassius*
(15) Carp, *Cyprinus*
(16) Fin whale, *Balaenoptera*
(17) *Horny fibre of shark*
(18) *Cattle*

Note: The only specimen not falling into line is No. 17. The chief protein of the shark fins is elastoidin which in many respects behaves extraordinarily, compared to the collagen of skin, e.g. by showing rubber-like behaviour after thermal shrinkage (Fauré-Fremiet, 1937; Fauré-Fremiet and Woelfflin, 1936; Champetier and Fauré-Fremiet, 1937). Elastoidin contains sulphur-bridges probably. It shows the Ewald reaction of extensibility also, which would be consistent with the presence of covalent cross-links (Fauré-Fremiet, 1937). Cf. Damodoran, M., *et al.* (1956) *Biochem. J.*, **62**, 621.

minor divergences in amino acids present in small amounts, such as serine, threonine and methionine. Hence the possibility of the hydroxy group of the hypro residue being involved in the intermolecular linking of collagen was obvious. The original view of the less-developed intermolecular hydrogen bonding on the peptide links of fish collagen had thus to be modified with due attention to the possible function of the hydroxy group in the interchain linking (Gustavson, 1953).

Fig. 1 shows the close straight-line relationship between the hypro content (in g. hypro per 100 gm. collagen) and the *Ts* of various fish-skin collagens (Takahashi and Yokoyama, 1954), the value of bovine skin being included for comparison. Evidently the hydrothermal stability of the collagen of skins of vertebrates is a direct function of their hypro content. In view of the fact that the available figures of the hypro have been estimated by an indirect method colorimetrically, a set of figures for the *directly* estimated contents of hypro and proline in the three types of collagen are given in Table I in gm.

TABLE I

CHARACTERISTIC PROPERTIES AND CONTENTS OF PROLINES OF COLLAGENS (GUSTAVSON, 1954a, 1955a)

No.	*Type of skin*	*Ts in ° C.*	*Per cent total nitrogen*	*Acid binding capacity in mmol. HCl/ gm. collagen*	*Per cent Proline*	*Per cent Hypro*	*Per cent Prolines*
1	Cod fish	40	18.3	0.92	10.8	6.0	16.8
2	Pike	55	18.4	0.93	13.3	7.9	21.2
3	Bovine (calf skin)	66	18.3	0.90	14.1	12.7	26.8

amino acid per 100 gm. collagen. The skin of cod fish represents the collagen of cold-water fish, the skin of pike the collagen of warm-water fish and the calf skin finally the mammalian (bovine) type of collagen.

Among collagen-like proteins in organism of invertebrates, the protein of the cuticle of the earthworm (*Allolobophora longa*) is perhaps best known in structural and chemical respects; the secreted type of collagen having mainly been studied from the cytological point of view. The cuticle protein which shows the wide-angle X-ray diagram characteristic of collagen (Reed and Rudall, 1948),

shrinks in water at 40° C., yielding a solution which does *not* gel on cooling.

The total nitrogen content of the dry cuticle is reported to be 14.5 per cent only. The protein contained 15 per cent of its total nitrogen in the form of hypro and only 3 per cent of proline, according to analyses by Mr. Singleton in Professor Astbury's laboratory which were kindly communicated by Professor Astbury, and reported earlier (Gustavson, 1955b). Hence in this instance, in spite of the great amount of hypro, the hydrothermal stability is low. However, it should be recalled in this connection that stretched gelatin gel, which gives the wide angle diffraction diagram of collagen, has its melting point in the range of 35-40° C. in spite of its content of hypro being the same as that of bovine collagen, with *Ts* of the order of 65° C. According to Reed and Rudall (1948), the earthworm protein is non-striated. In view of the non-gelling of the solution of the melted cuticle protein and with due consideration of the aforementioned facts regarding gelatin and non-striation, it seems doubtful whether the earthworm protein should be considered to be a member of the collagen family.

Watson and Smith (1956) have carried out a complete analysis of the amino acids present in the earthworm cuticle, which contained 14.6 per cent nitrogen, equivalent to about 80 per cent of collagen. The contents of glycine and non-polar amino acids were practically identical with those of bovine collagen. The proline figure was only 1.2 per cent of the total protein nitrogen, while the hypro was present in such a large amount as 12.6 per cent. Thus, earthworm cuticle contains a higher proportion of hydroxyprolyl residues than any collagen yet analysed. Also the aliphatic hydroxyamino acids are higher in the cuticle protein than in the bovine collagen, whereas the contents of lysine and arginine are appreciably lower. The protein sulphur is appreciably lower than that of bovine collagen (<0.1 per cent S). Evidently, additional information on the fine-structural details is desirable before any theorization on the implication of the hypro content — hydrothermal stability is justified in this particular instance.

In this connection it should be noted that the reptile skins which are commercially used, such as the skin of the alligator and the crocodile, shrink at 60-63° C. The lizard skins of commerce have *Ts* of 62-65° C. The degree of structural stability of the snake skins of importance in leather manufacture is of the same order. It is in-

teresting to note that the skin of the Australian lung-fish belongs to the mammalian type of skin as far as hydrothermal stability is concerned (*Ts*: 65-67° C.).

Obviously, the data in Fig. 1 and Table I are no evidence in themselves for the view that the hypro residue is directly involved in the stabilization of collagen; for example by forming a site for an interchain hydrogen bond. The effect of the irregularity of the polypeptide chains imposed by the prolines may be indirect and even fortuitous.

However, if collagen is mainly stabilized by interchain hydrogen bonds on oppositely located keto-imide groups of adjacent chains, the content of pyrrolidine residues should be a factor of importance. The residues of the prolines which are built into the polypeptide chains with the formation of —CO.N— links introduce a regular interruption of the normal —CO.NH— links, formed by the remainder of the amino-acid residues. Accordingly, sites for the compensation of adjacent —CO— groups of the keto-imide links are withdrawn, which implies fewer stabilizing cross-links and consequently a lowered degree of stability. Then, mammalian collagen should be less stable to heat than fish collagen. However, the reverse is true.

INTERCHAIN HYDROGEN BOND BETWEEN HYDROXY AND KETO-IMIDE GROUPS

On the other hand, supposing that the hydroxy groups of the residues of the hydroxyamino acids may enter into interchain hydrogen bonding with some other groups, for example, the carboxyl, or the carbonyl of the —CO.NH— linkage, the stability of the structure would be expected to increase with the content of hydroxyproline as is actually found.

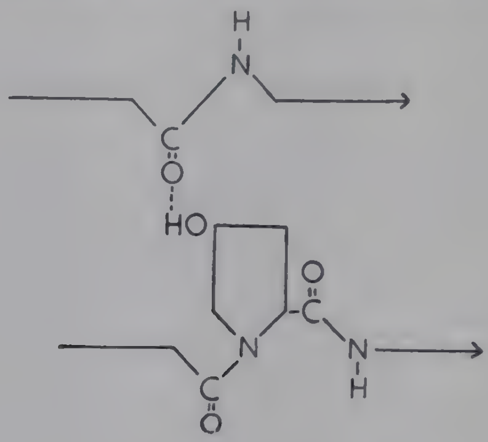
A satisfactory explanation of the correlation of the hydroxyproline content of collagens and their *Ts*, and indications of the type of link formed, have been arrived at by investigations of the behaviour of exhaustively acetylated bovine collagen (Gustavson, 1954b; 1955c). Such a collagen with all its amino-groups (0.39 mmol./gm. collagen) and 80 per cent of its hydroxy groups inactivated (1.39 mmol./gm. collagen) was obtained by acetylating hide powder and calf skin, according to the method of Green *et al.* (1953). Comparative experiments with N-acetylated collagen were included. The hydro-

thermal stability of collagen was not affected by its N-acetylation, while the N- and O-acetylation, that is, the blocking of the hydroxy- and amino-groups, lowered the *Ts* from 64-66° C. to 40-44° C. (Gustavson, 1954a, b; 1955a). This finding indicates the rupture of *interchain* cross-links by the O-acetylation.

Assuming that the aliphatic hydroxy groups are preferentially acetylated, at least 0.8 mmol. of hydroxyproline per gm. collagen should have reacted. Hence, out of 1.07 mmol. hydroxyproline residues of the original collagen, three out of four hydroxy groups have been blocked. In this connection, it is of interest to note that *Ts* for the O-acetylated bovine collagen coincides with that of the skins of most cold-water fishes, suggesting that the hydroxy group, resisting acetylation, forms a stronger bond than the rest of the hydroxy groups, and further that this particular group is mainly responsible for the stabilization of the teleost collagen.

From investigations of the effect of the inactivation of the hydroxy groups of collagen on its reactivity, the results from series with condensed vegetable tannins (mimosa) and non-ionic complexes of sulphito-chromium sulphates are most instructive (Gustavson, 1955c). The fixation of these compounds by collagen is appreciably increased by its heat-denaturation, and by its pretreatment with lyotropic agents, indicating that the irreversible fixation of these compounds by collagen involves non-ionic protein groups (hydrogen bonding) (Gustavson, 1926a). The mimosa tannins are mainly fixed by hydrogen bonding of the polyphenolic structures on the carbonyl oxygen of the keto-imide group (Gustavson, 1946a; 1947a; 1950a; 1954c, e; Lanham and Pankhurst, 1956). The very marked affinity of the condensed tannins for modified polyamides, which contain the $-$CO.NH$-$ linkage as the principal site of binding, is the crucial evidence (Gustavson, 1954c, e). It is important to note that the non-ionic chromium complexes are not bound by the polyamides (Gustavson, 1954d; 1955c). Hence, they are probably not reacting with the keto-imide linkage of collagen. Accordingly, the increased fixation of these chromium complexes by heat-denaturation of collagen and by its pretreatment with lyotropic agents (urea, for example) cannot be due to the freeing of co-ordination sites on the $-$CO.NH$-$ linkages. By O-acetylation of collagen, the irreversible binding of mimosa tannins by collagen is greatly increased, from 60 to 95-105 per cent, on the basis of protein. On the other hand, the fixation of the non-ionic chromium complexes is

markedly impaired by the blocking of the hydroxy groups by acetylation; decreases of the order of 30 per cent being commonly found (Gustavson, 1955c). These findings with due regard for other facts which cannot be enumerated here, are in harmony with the view that a part of the hydroxy groups is linked by a hydrogen bond to the keto-imide group, schematically, as:



The rupture of such a bond by heat denaturation (Gustavson, 1955a), or by the lyotropic pretreatments mentioned, should satisfactorily explain both the increased fixation of vegetable tannins (by the freed –CO.NH– bond), and of the non-ionic chromium complexes (by the freed OH-bond) on heat denaturation of collagen, as well as the effects of the blocking of the hydroxy group by acetylation, that is, the impaired hydroxy-coordination of the chromium compounds, on the one hand, and the increased binding of the keto-imide-attached polyphenols, on the other. The large decrease of *Ts* recorded ($\triangle Ts = -25°$ C.) is also explained. In view of the marked affinity of the native collagen for the agents mentioned, it is probable that only a few of the hydroxy and keto-imide groups are participating in this inter-chain cross-linking.

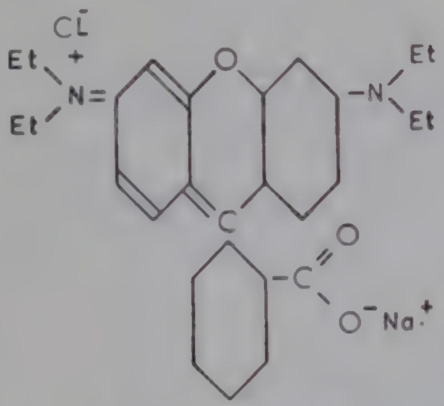
The following considerations suggest that the hydroxy group involved is mainly furnished by the hydroxyproline residue. In the presence of an aliphatic OH-group in intermolecular cross-linking, the teleost collagen should form a more stable structure than the bovine type, since the fish collagen contains more such hydroxy groups than the bovine collagen. In view of the reverse trend of the shrinkage temperature, aliphatic hydroxy groups are not likely to be involved to any great extent (Gustavson, 1955a). The importance of other types of bonds for the cohesion of the collagen structure, including the common type of hydrogen bond and more stable

links, is by no means questioned by this accentuation of the function of the bond on the hydroxy group. (See Gustavson, 1955b; 1956a).

CHEMICAL EVIDENCE FOR THE $-\mathrm{OH}---\mathrm{OC}\langle^{\mathrm{NH}-}$ CROSS-LINK

Additional evidence for the existence of an intermolecular bridge in the form of a strong hydrogen bond between the hydroxy group and the keto-imide group has been produced by investigating the fixation of various chromium complexes by collagen and by acetylated and otherwise modified collagens which show that part of the binding of non-ionic chromium complexes by collagen is located to the hydroxy groups which are more or less blocked in the native collagen. Reference can only be given to these investigations since the main aspects and the details of the procedure fall outside the provinces of the present paper (Gustavson, 1955c; 1956b).

The behaviour of a number of aromatic compounds to collagen has been included in this study. In this connection some unpublished data from the fixation of the zwitter-ionic dye Rhodamine B and the substantive dye Benzopurpurine 4B will be briefly discussed. Rhodamine B is a xanthene derivative and, as seen from its structure, it may be regarded as fluorescein in which the two hydroxyls are replaced by diethylamino groups.



The rhodamine molecule carries both positively and negatively charged groups and hence, it is an ampho-ionic dye. It shows strong hydrogen bonding with the hydroxy group and with other hydrogen donor groups which most strikingly has been demonstrated by Lundgren and Binkley (1954) who measured the change of the colourless rhodamine to the coloured zwitter-ionic form produced by hydrogen donor compounds. By the incorporation of the

propiolactone polymer into wool, structures of this type: $H(OCH_2 \cdot CH_2 \cdot CO)_n OCH_2 \cdot CH_2 COOH$ are formed and fixed by keratin (Lundgren, 1954; Jones and Lundgren, 1951; Rose and Lundgren, 1953). The ability of the wool to fix the rhodamine is more than doubled by this pretreatment. This increased reactivity is mainly due to the donor hydrogen function of the hydroxy group introduced. It supplies direct and unequivocal evidence for the formation of hydrogen bonds on the OH group. Some data on the fixation of Rhodamine B by intact collagen and by modified collagens may therefore be of interest. Portions of 500 mg. of collagen, both intact and modified specimens, were equilibrated with 25 ml. portions of the dye solution (0.04 per cent) at pH 5. In one series, the total uptake of the dyestuff was obtained by estimating the content of the dye left in the solution at attained equilibrium colorimetrically by the Spekker absorptiometer. In duplicate series, the irreversible fixation of the dye by the various preparations of collagen was estimated indirectly colorimetrically on the combined filtrate and washings (500 ml. total volume).

Data of the amount of the dye removed by the various preparations of collagen and the amount of dye irreversibly fixed are given in Table II.

Apparently, in the reaction of collagen with Rhodamine B which

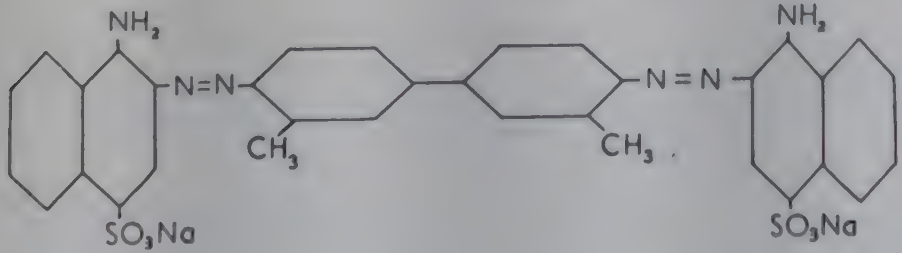
TABLE II

THE IRREVERSIBLE AND REVERSIBLE BINDING OF RHODAMINE B BY COLLAGEN (in per cent on the weight of collagen)

No.	*Type of substrate*	*Total uptake of dye*	*Irreversibly fixed dye*	*Reversibly held dye*
1	Ordinary hide powder (= H.P.) Blank	0.20	0.12	0.08
2	Denatured H.P.	0.29	0.16	0.13
3	N-acetylated H.P. (0.39 meq. acetyl per g. collagen)	0.23	0.12	0.11
4	Exhaustively acetylated H.P. (N+O); (1.58 meq. acetyl per g. collagen)	0.28	0.16	0.12
5	Deaminated collagen (0.30 meq. amino groups per g. collagen removed)	0.21	0.12	0.09
6	Native steer-hide hide powder (unlimed)	0.17	0.12	0.05
7	Cod-fish skin (native), as powder	0.28	0.07	0.21

is known to react with a number of different protein groups, various protein groups, ionic as well as non-ionic, are involved. This versatility of reaction and its non-specificity makes this dye less suitable for demonstrating the specific function of any individual group, for instance the hydroxy group of the collagen. This is particularly the case with the exhaustively acetylated specimen of collagen, which shows a markedly increased uptake and fixation of the dye, while the rather extensive inactivation of the hydroxy group of collagen by acetylation should be expected to impair the uptake of the dye drastically, since it possesses strong affinity for the OH-group, as strikingly demonstrated by Lundgren and Binkley (1954). As already mentioned, the hydrogen bonding capacity of collagen towards polyphenols is increased by the exhaustive acetylation. The large swelling of the collagen which takes place on acetylation connotes the breaking of intermolecular cross-links and concomitantly new *loci* for co-ordination (hydrogen bonding) are formed. Hence, for an unequivocal demonstration of the function of the hydroxy groups of collagen, a modified collagen in which only the hydroxy group is changed, should be the suitable substrate. Such a modified collagen is not obtainable yet. As to the other substrates in Table II, a direct comparison of all the specimens except Nos. 3, 4 and 5, can be made. It is noteworthy that native bovine collagen fixes about twice the amount of the dye which is fixed by fish collagen *irreversibly*, whereas the cod-skin collagen *sorbs* about four times as much of the dye as the bovine collagen. By heat-denaturation of mammalian collagen both types of the uptake of the dye by collagen are increased. Cf. Lundgren and Binkley, 1955.

Among the dyes tested, only one will be mentioned, namely Benzopurpurine 4B, which is represented by the following structural formula:



It is a substantive dye which shows a marked affinity for substrates containing hydroxy groups, for instance, cotton and cellulose (Valko, 1937). In the pH range corresponding to the isoelectric

zone of collagen, e.g. pH values about 5, the binding of this dye apparently occurs mainly by the OH groups of the substrate, the ampho-ionic character being less apparent. As an example, the following set of experiments is given. Portions of hide powder equal to 500 mg. of collagen were shaken for 24 hours in 20 ml. portions of 0.01 per cent solution of the dye (= 2 mg. dye). The filtrates were drawn off and the stock washed and shaken three consecutive times with 40 ml. of water. The filtrate and the washings were made up to 200 ml. The amount of unfixed dye was determined colorimetrically in the Spekker absorptiometer. The amount of dye removed by the substrate was obtained by difference. The irreversible fixation of the Benzopurpurine 4B was for ordinary hide powder 0.40 per cent on the weight of collagen, for the same hide powder after heat-denaturation (for 1 minute in 70° water) 0.48 per cent and for the exhaustively acetylated hide powder 0.17 per cent only.

The irreversibly fixed dye was stable to saturated solution of phenol upon prolonged treatment at room temperature, whereas 6–8 molar solutions of urea stripped the dye almost completely upon two consecutive extractions at 20° C. These findings indicate hydrogen bonding to be chiefly responsible for the attachment of the dye to the collagen.

These data indicate the importance of the hydroxyl groups for the non-ionic reactivity of collagen. They provide also evidence for the existence of a part of the hydroxy groups internally compensated which makes them less reactive. By denaturation these cross-links are severed and the increased reactivity of the denatured collagen to hydrogen-bonding agents may be conceived to be due to the new sites of hydrogen bonds which are set free by breaking the $-\mathrm{OH}---\mathrm{OC}\big\langle^{\mathrm{NH}-}$ cross-link.

HIGH CONCENTRATION GRADIENTS SET UP AT THE INTERFACE: COLLAGEN-SOLUTIONS OF ELECTROLYTES

In elucidating the reactions in a two-phase system, such as collagen in aqueous solution, it is generally considered that for systems in which the membrane potential (Donnan effects) have been eliminated, or do not exist (as in irreversible systems), the concentration of the solute in the internal solution of the solid phase is equal to the

concentration of the solute in the external solution. Results of recent investigations of gelatin gels (Jelley and Pontius, 1955), reacting with solutions of acid dyestuffs, and experimental studies on the fixation of cationic chromium complexes by collagen from solutions of basic chromium chlorides (Gustavson, 1957) show to all evidence that this assumption is not valid. In fact, very large concentration increases are noted. Thus in a recent paper, Jelley and Pontius (1955) report that the equilibrium concentration of the dyes, for instance the simple acid dye represented by the sulphonic acid of amino-naphthalene-diazotoluene, is enormously greater in the surface of the gelatin gel than in the dye bath. The concentration in the gel phase may be some two hundred times greater than in the dye bath. The setting up of similar concentration gradients in systems such as that of weak acids and salts with collagen may be of physiological importance, for instance for the connective tissue. The lyotropic effect of the concentrated solutions (Gustavson, 1926a) may profoundly affect the collagen, modifying its properties and reactivity, a possibility not heretofore realized.

Additional evidence for this accumulation of solute in the interface is provided by data from the fixation of cationic complexes of basic chromium chlorides by collagen (Gustavson, 1957). A solution of the 67 per cent acid chromium chloride was equilibrated at a concentration of 1 equiv. Cr per litre. Its composition corresponded to the empirical formula: $Cr_2(OH)_2Cl_4.2NaCl$. By ionic exchange, the chromium was found to be entirely positively charged (Gustavson, 1924; 1944; 1946b). All the chlorine was found in the filtrate from the cation exchanger. Hence, *no* complexly bound chlorine was present. In the original stock solution at a concentration of 250 gm. Cr_2O_3 per litre, an amount of 5 per cent of the total amount of chromium was present as non-ionic complexes. The figures for the acidity of the complexly held Cl-groups (*chloro*-groups) varied widely in the range from 15 to 30 per cent. In view of the lability of the chloro-groups (on dilution), this large spread of the values is understandable. It is indicated that the solutions of great concentration of the chromium chloride consist mainly of complexes containing two Cl-groups in the binuclear complex (2 atoms of chromium), i.e. it is the 67 per cent acid dichloro-diol-chromium chloride $(\underset{Cl}{Cr}\overset{OH}{\underset{HO}{\rightleftharpoons}}\underset{Cl}{Cr})\,Cl_2$. On tenfold dilution, the chlorocomplex

is evidently rapidly and completely decomposed; the final product being the $\left(Cr\begin{smallmatrix}OH\\HO\end{smallmatrix}Cr\right)Cl_4$ compound.

The entrance of Cl-groups into the chromium complex by the augmentation of the *chrome concentration* is an item of particular importance for the experiment to be discussed. Portions of hide powder were equilibrated with the aged solution of the 67 per cent acid chromium chloride containing 25 gm. Cr_2O_3 per litre, in which *no* chloro complexes were present. This treatment was repeated; the total duration of the tannage being 8 days, with final pH values of 2.9-3.0. The chromed hide powder had its binding capacity for these chromium complexes completely satisfied. It contained 4.10 mg. equiv. of Cr and 2.12 mg. equiv. hydrolysable Cl per gm. collagen. By equilibrating the tanned hide powder, after removal of uncombined chromium salt by washing, at pH 8.2 (4 per cent pyridine solution) and at pH 5, the distribution of the bound chloride was estimated. The Cl-groups may be present as (1) protein-bound acid (HCl); (2) complexly bound in the chromium complex (*chloro*-groups) which are stable to the treatment with 4 per cent solution of pyridine on 1-2 hours' duration; and (3) Cl-groups present in electrostatic compensation with the unipointly fixed chromium complexes ('Gegen-ion'). The original chromed hide powder contained 2.12 mequ. Cl. per gm. collagen, which was distributed as follows (in mequ. per gm. collagen): 0.86 protein-bound Cl; 0.55 Cl as 'Gegen' ions (associated electrostatically with Cr) and 0.71 as complexly bound Cl (*chloro*-groups). The composition of the fixed cations corresponded to the formula $(Cr_2(OH)_3Cl)<$, or an acidity of 17 per cent.

Some intricate and puzzling inconsistencies and discrepancies of old standing are given a satisfactory explanation by this new conception of the reaction. Far back in the 'twenties, Stiasny and Balányi (1927), and the present author (1927b) found no complexly bound chloride in dilute solutions of the 67 per cent acid chromium chlorides of the type employed (prepared by the addition of one mole of NaOH to one mole of the Bjerrum salt $(Cr\,Cl_2(OH_2)_4)Cl.$-$2H_2O$). The first-mentioned workers employed concentration chain measurements of the chloride ion and the last-mentioned investigator the ion exchange technique. However, by analysis of the hide powder, tanned with the same solution of the basic chromium chloride which was found to be free from chromium complexes with

complexed Cl, by means of the pyridine method as well as by the diffusion-neutralization method, chloro groups were found (Gustavson, 1926b; 1927a; 1931; Küntzel *et al.*, 1934).

No reasonable explanation of the repeated findings of chloro-chromium complexes in collagen tanned with basic chlorides free from chloro-chromium complexes, has been found, nor of the preferred fixation of cationic complexes of the type $(Cr(OH)_2Cl)<$ by collagen, on the basis of the available experimental material and the concept of the mechanism of the reaction generally accepted. However, in view of the formation of chloro-chromium cations in solutions of basic chlorides being favoured by increasing the concentration of the compound (Gustavson, 1927b; 1944) and moreover, with due consideration of the tremendous enrichment of the concentration of solutes at the boundaries of the solid protein-aqueous solution found in the investigations by Jelley and Pontius (1955) just referred to, this apparent paradox is removed. By the increased concentration of the chromium compound in the protein, chloro-diol-chromium cations may be formed which in this form combine with collagen.

This example may appear to be rather far-fetched and not to belong to the problems under discussion in the present symposium. However, some excuse is the fact that it provides a demonstration and a proof of the setting up of very great concentration levels of the reacting solute at the interface between the collagen and the aqueous solution. It constitutes evidence for a phenomenon that is likely to enter prominently into the behaviour of collagen and connective tissue. By employing the tracer technique, this interesting phase of the reaction of collagen and solid proteins generally with electrolytes could probably be advantageously attacked.

THE POSSIBILITY OF '*IN VIVO*' TANNING OF COLLAGEN

Very little positive information is at hand concerning tanning processes in organs of higher vertebrates including man, although the importance of such hardening of protein structures of invertebrates is firmly established. For a general review, see Gustavson (1956b). Modifying of the proteins of connective tissue by self-tanning is one possibility. Thus, by splitting-off of certain reactive groups of collagen, such as strongly polar side chains, the hydration of

the structure should be lowered, and the stability of the structure should be expected to be improved. On the other hand, the elimination of the residues of the amino acids which are instrumental for the cross-linking and organization of the protein, such as the hydroxyproline residue, would be expected to have the reverse effect on collagen, i.e. impair its stability. It appears that the abnormal collagenous tissue formed in rheumatoid arthritis is low in hydroxyproline. This degenerated collagen shows a lower degree of stability and is also more soluble in alkali than ordinary collagen. In view of the importance of the hypro residue for the structural stabilization, as evident from the discussion in the foregoing sections, it would appear that *in situ* alterations of collagen affecting the particular chain in the trihelical protofibril in which the hypro-residue is laid down primarily, for instance by partial removal of such a polypeptide chain, should markedly impair the stability of the protein structure (Cowan *et al.*, 1955; Burton *et al.*, 1955; Reed *et al.*, 1956).

The properties of collagen may be modified also by cross-linking *in situ* by a secondarily formed group, possessing tanning and cross-linking potency, in the polypeptide chain without removal of the side chain. Thus, the residues of the aromatic amino acids are able to take on a quinoid character, thereby creating potential cross-linking facilities. This type of self-tanning appears to be of wide occurrence and of great importance among arthropods (Blower, 1947; Brown, 1950; Dennell and Malek, 1956). Such alterations by cross-linking and hardening of the protein structure would make it more rigid and resistant. However, it would concomitantly imply a lowered degree of elasticity which latter impairment would be disastrous for the primary function of the tendon, for instance.

The occurrence of the ordinary modes of tanning for modifying the properties of collagen in tendon and connective tissue generally is a possibility which should not be altogether ignored. In the first place, the partaking of tanning and hardening by aldehydes and unsaturated fatty acids, as well as by steroids, merits attention, as first pointed out by Rudall (1946) in discussing the hardening of epidermin and protein structures of insects. Pathological alterations of collagen and changes on ageing of the body may well involve cross-linking of the protein by aldehydes such as methylglyoxal (pyruvaldehyde), an intermediate in the metabolism, and by highly conjugated glycerides or fatty acids or their peroxides. Increased

formation of such compound may occur on disturbance of the enzymatic processes. The optimum reactivity of these compounds for collagen is located at the pH value of living tissue. Purely speculatively, the similarities between the effects of cross-linking of fibrous proteins (tendon) by aldehydes and the alteration which tendon undergoes on ageing of the human body has been mentioned (Gustavson, 1947b). In both instances, the changes are alike: the degree of hydration and the water-retaining capacity of the structure are reduced. Further, the degree of elasticity of the tendon is markedly impaired.

In this connection some findings in Stucke's investigation (1950) of the tensile strength and degree of elasticity of the human Achilles tendon from autopsy of healthy and diseased cases are of immediate interest. The maximum tensile strength of this tendon is attained at the age of 20-30 years in healthy humans; the maximum zone being followed by a rapid decrease in the functional properties of the tendon. In tendons from individuals who had died from certain severe diseases, the function of the tendons was greatly impaired. The extension diagrams and the stress-strain curves obtained by Stucke from these pathological cases and from senile cases differed strikingly from those of the tendons of healthy young humans. Actually, these diagrams closely resemble the diagrams which are given by normal tendon after its tanning with aliphatic aldehydes, such as formaldehyde, glyoxal and methylglyoxal. There are probably several causes for the tendon approaching the amorphous state of structure, such as dehydration, lessened water-retaining capacity, and deposition of organic and inorganic material. Cross-linking by methylglyoxal, for instance, has been mentioned as an additional factor (Gustavson, 1947b). Since very minute amounts of this aldehyde only will suffice for extensive stabilization of the collagen lattice and to modify the protein profoundly, it would be very difficult to prove the case by chemical analysis. However, it appears that the possible occurrence of such processes would be worthy of the attention of the medical specialist.

SUMMARY

The importance of the hydroxyproline (= hypro) residue for the stabilization of the collagen structure, particularly as to its hydrothermal stability, is discussed in the light of new experimental data

and in relation to recent models of the collagen helices. It has earlier been experimentally indicated that the hydrothermal stability of the skins of various mammals and fishes, as measured by the temperature of instantaneous shrinkage of collagen, is augmented by increased content of hypro. Thus, collagen of teleostean skin shows low content of hypro (6-10 per cent) and low values of the shrinkage temperature (35-55° C.), whereas the collagen of mammalian skin contains larger quantitites of hypro (12-13 per cent) and markedly higher shrinkage temperature (60-70° C.).

The effect of exhaustive N- and O-acetylation of the collagen of bovine skin on its shrinkage temperature and on its affinity for and binding capacity of various agents of ionic as well as co-ordinate reactivity is discussed. The results are interpreted as indicating the presence of a comparatively strong intermolecular hydrogen bond between the hydroxy group of the hypro residue and the oxygen atom of the keto-imide group on an adjacent polypeptide chain. Data on the behaviour of agents with specific affinity for the keto-imide link and for the hydroxy group of collagens are considered as well as the effect of hydrothermal denaturation of collagen upon its reactivity.

The building up of highly concentrated solutions of the ionic reactants at the interface of the solid phase in the two-phase system of collagen-solutions of electrolytes is indicated by data from the reaction of basic chromium chlorides with collagen. The changes in the complex composition taking place in the cationic chromium complexes on their fixation by the hide protein, as compared to the composition of these complexes in the external solution, provide evidence for the enrichment of the concentration of the solute on the surface of collagen. From the concentration profile of gelatin gel on its fixation of sulphonic acid dyestuffs, a drastic jump in the concentration of the dye at the interface of the gel is also found. Such concentration gradients might be set up on the reaction of collagen with dilute solutions of electrolytes, such as weak organic acids, under physiological conditions, creating conditions favourable for hydrotropic effects on the protein component.

Further, the possible occurrence of changes in collagen by ageing, or under certain pathological conditions, due to the formation and binding of aldehydes, such as pyruvaldehyde and similar compounds by collagen (tendon) is suggested to be worthy of consideration.

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GROUP DISCUSSION

DR. BEAR drew attention to the X-ray diffraction differences between the collagens with 'abnormal' T_s and those with normal T_s. Earthworm cuticle gives the collagen wide-angle diagram and must, therefore, have the 3-chain coiled-coil molecular structure but it has no large-scale order in evidence at small angles. Elastoidin gives both wide-angle and small-angle collagen diagrams but, as Champetier and Fauré-Frémiet showed, it behaves more like an aldehyde cross-linked collagen in showing reversible heat shrinkage. He wondered whether such stabilization might occur within the collagen fibre as a result of enclosure in the tissues surrounding it. On drying the elastoidin fibre, the small-angle diagram shows considerable evidence of intrafibrillar distortion indicating less thermal stability of the elastoidin fibrils than the whole tissue results suggest.

DR. GUSTAVSON, referring to work of Fauré-Frémiet in 1937, thought that cross linkages involving sulphur might occur in elastoidin, which then should be a slightly vulcanized protein, to use Dr. Astbury's term.

DR. BEAR said that in view of the recent 3-chain models for collagen molecular structure, one should discriminate between *inter*molecular cross linkages and interchain *intra*molecular cross linkages. One of the molecular models allows intramolecular bonds involving hydroxyproline more readily than another which favours intermolecular bonds. The latter seems to be in better agreement with the diffraction data. Workers now consider that the gelatinization of molecularly dispersed collagen should be studied and attempts made to see if there is any correlation of hydroxyproline content with the thermal shrinkage temperatures of these molecules themselves as well as with thermal contraction temperatures of the tissues.

DR. GROSS said that it was difficult to correlate the properties of all collagenous fibres with their structure as they are probably often not simple proteins but mixtures. For example, trypsin-purified elastoidin has a tyrosine content of 6 per cent. After autoclaving, the gelatin dissolved out contained 1 per cent tyrosine but there was an insoluble residue con-

taining 25 per cent tyrosine; obviously a non-collagenous protein and perhaps strongly bound to the collagen. Again, carp-skin collagen contains 8 per cent hydroxyproline and is insoluble in dilute acetic acid, whereas carp-swimbladder tunic with a higher hydroxyproline content (11.9 per cent) is soluble in dilute acetic acid. Both these tissues yield gelatins with identical hydroxyproline contents. The difference in solubility may be due to differences in the closely associated non-collagenous contaminant.

Dr. Gustavson said that intermolecular cross-links might be formed under favourable steric conditions. Although gelatin has the same amino acid composition as collagen, including the same amount of hydroxyproline and upon stretching gives the high-angle X-ray diagram typical for collagen, it does not acquire the physical properties of collagen by the orientation. The stabilizing intermolecular links, as those joining the trihelical ultimate units by the hydrogen bond formed by the OH of the hydroxyproline residue and the carbonyl oxygen of an adjacent keto-imide group, are ruptured in the transformation of collagen into gelatin. The bonds are not reformed by stretching the gelatin, probably due to unfavourable steric conditions. A similar explanation may be given to the irregularity shown by the protein of the cuticle of the earthworm as to its hydroxyproline-shrinkage temperature relationship. Differences in the fibrillar structure are probably involved also.

Dr. Bowes asked Dr. Gustavson if he could suggest any reason for the greater reduction of T_s of collagen caused by treatment in calcium hydroxide solutions compared with sodium hydroxide solutions of the same pH. The T_s was reduced from 60° to 45° in 7-8 days. The T_s was determined at pH 5 to 6.

Dr. Gustavson thought it was a specific effect of the calcium and referred to the research of the school of Carl L. A. Schmidt on the binding of calcium by the hydroxy groups of proteins and to Marriott's work on the alkali swelling of collagen.

Dr. Bowes suggested that removal of non-collagenous protein which also occurs during the alkaline treatment might also be important.

Dr. Reed thought that the low shrinkage temperature of the fibrils of the earthworm cuticle might be due to the different structural organization in this tissue. The fibres are very loosely spaced and might, therefore, be more accessible to water.

Dr. Meyer asked if tanning by tannic acid involved oxidation.

Dr. Gustavson said that this was only true at very high pH values but not on the acid side of the iso-electric point of collagen, the range in which tanning is usually performed.

Dr. Fitton Jackson had found the solubility of earthworm cuticle in dilute acid to be much less than that of vertebrate collagen. Even when

it was taken into solution it had not been possible to precipitate it in the same way as vertebrate collagen and (as Dr. Bear had mentioned earlier) it would not form gelatin. It was also found to contain 20 per cent of tightly bound polysaccharide, which might account for the difference in the solubility properties.

THE AGEING OF COLLAGEN

F. Verzár

I should like to apologize for returning to macroscopic observations at a time when X-ray and electron microscopy have contributed so much to our understanding of the structure of connective tissue in general and of collagen in particular. I hope that the present work will serve at least as a model for the conclusions reached by modern physico-chemical methods.

It seemed to me important for the study of natural collagen to choose a simple source of material, and if possible one not mixed up with other tissue ingredients. Whole tissues, like skin, contain several similar substances, collagen fibres, elastic fibres, argyrophil fibres, ground substance and probably other intermediary substances. Further, since we now know that these substances can be transformed into each other (Burton *et al.*, 1956), there is the risk of transforming artificially one into the other, e.g. when preparing collagen-free elastin. Simpler methods may, therefore, sometimes have advantages. Thus we turned to the single fibre of the rat's tail tendon (Verzár, 1955). Ewald, as early as 1909, used mouse tails, Partridge (1948), Banga (1953) and others have used the rat's tail, Weir (1949), Weir and Carter (1950) and many others kangaroo-tail tendons. We have used an even more simple structure: the single tendon fibre. In the fully grown adult rat these fibres have a remarkably constant diameter and sub-macroscopic structure and are therefore especially convenient for comparative work.

These tendon fibres have to transmit the pull of the muscles proximal to the base of the tail, to the distal joints of the tail. Therefore, they should not be extensible. They are what one might call with all due respect for a complex protein structure, pure collagen fibres.

From the tip of the tail, through a small hole in the skin, one can extract (even from the living narcotized animals and without bleeding) single tendon fibres which are about 10 cm. long, have a diameter of 0.15 mm. and a weight of 4-5 mg. This is our material. That it is a single unit is controlled not only by the above three characteristics, but also by examination under the microscope where

it shows a beautiful helical structure with small angle episcopic illumination.

It is curious that the periodic or helical structure of collagen fibres, so well seen under the electron microscope and also deduced from X-ray analyses, has not been recognized in the microscopic field or at least is not recorded in the modern textbooks, although occasional reference is to be found in the older literature. Woerdemann (1921) described helices in the mouse tail tendons, and Heringa (1926) with his co-workers described them in skin and in tendons. He spoke of 'spiral arrangements'. His conclusions were drawn from hygroscopic torsions of bundles of tendons and from other optic phenomena. Nauck (1931) working on whole human and other tendons saw 'waves' and 'periodical double lines, 13 per mm.' Heringa's measurements were similar. Lerch (1950, 1953) spoke of the macroscopic structure of tendons, which is like a cable with large helical windings. The helical structure can not be seen with transparent illumination. It can easily be demonstrated if the tendon is placed in Ringer's solution on a slide and illuminated episcopally under a small angle.

With a double episcopic low angle illumination and especially with two lamps with lights of different wave-lengths (MacConaill, 1955), the helical structure can be demonstrated in different colours.

With a single episcopic illumination the helices are best seen if the beam of light falls on the fibre at an angle of 90°. Turning the slide, the helices disappear completely at 180°.

The microscopical helical structure of neighbouring tendon fibres lie in different planes. This makes it possible to recognize whether one is dealing with a single fibre or a bundle of two or more. Bundles of fibres are again helically twisted around each other like a cable (Fig. 1).

These thin fibres lose their water by evaporation extremely quickly even during their preparation. Dried fibres show no helices, but they appear immediately if the fibre is transferred to water or to a salt solution.

The analogy of this helical structure with the descriptions based on X-ray analyses (Astbury and Sisson, 1935; Bear, 1953; Springall, 1954; Hall *et al.*, 1955; Randall, 1954; Robinson and Watson, 1952; Ramachandran and Kartha, 1954) and electron microscopic pictures is striking. Of course, it must be realized that we are working here in an order of magnitude several thousand times larger. One may

say, however, that it seems that similar principles underlie the organization of tendons and the molecular arrangement of collagen fibres.

A curious observation must be mentioned here also. The helical structure disappears with very great loads or stretching, but it reappears on unloading, and this can be repeated indefinitely. Thus a certain helical molecular orientation is disturbed by stretching, but is completely reversible.

Thermic contraction of these single tendon fibres has been used to study mechanical and microscopic changes and to relate these observations to each other.

Our method has been described previously (Verzár, 1955). We used a carefully controlled water-bath. Tendons were immersed in Ringer's solution, i.e. isotonic with blood and at pH 7.4, mixed with oxygen with 5 per cent CO_2 to keep the pH constant. A cathetometer served for continuous measurement of the fibre length. All fibres were 50 mm. long and 0.15 mm. broad, and loads from 100 to 10,000 mg. were attached to the free end. Experiments were made by immersion in Ringer's solution at 40° C with heating up to 70° C. In other series the tendon was immersed in a solution at a constant temperature of 58° or 60°, etc.

The microscopic changes seen during thermic contraction are recorded in Fig. 2. (They were also shown in a cinematograph film.)

After immersion with a load of 200-500 mg., which does not overstretch the fibre, the helical structure can be well seen, photographed or cinematographed. After heating to 62° C, a thermic contraction appears, the fibre shortens to about 40 per cent of its original length and becomes several times thicker. The helices seem to be squeezed together and can be seen in fine lines.

If at this stage, that is before the thermic contraction has reached half of maximum, one transfers the fibre quickly to a cold Ringer's solution, it can be stretched to the original length, and the broken structures of the helices can still be seen under the microscope. Such a fibre is partly extensible. If it is again heated to 62° C, it will give again a thermic contraction until it reaches its maximal shortening.

If we continue the heating after the maximal shortening, the helical substance disappears completely and the fibre becomes transparent and glassy (Fig. 2*d*, *e*, *f*). At that moment it is completely elastic, in contrast to the natural fibre. The thermic contrac-

tion is only partly 'reversible' as the fibre is now 'rubber elastic', and even a small load extends it again. This is, therefore, not a true reversibility. These fibres do not give a second thermic contraction.

If the temperature reaches 68-70° C, all fibres break.

So far, all this is in agreement with established facts. The unelastic collagen fibre is transformed into a second stage of elastic or elastoid fibre. The latter shows no helical or periodic structure; it is digested by elastase and gives the histological staining of elastic fibres and shows only an axial longitudinal structure.

The helical band of the fibre must be imagined as a plane structure going straight through the whole diameter of the fibre. This can be demonstrated with dilute acetic acid (0.1-0.01 per cent) at room temperature. This dissolves the substance of the helical structure. It diffuses from the outside towards the centre, and one can observe directly that the fibre centre still contains helical substance, after it has disappeared at the periphery.

If one keeps a fibre in 0.1 per cent acetic acid at room temperature (20° C), the fibre becomes completely transparent without contracting. One has to transfer it to a hot fluid to get a thermic contraction. However, direct measurements showed that if the natural fibre gave a thermic contraction at 62° C, with the addition of acetic acid, at about pH 6, the thermic contraction will occur at about 45° C. Thus destruction of the helical substance by acid enhances the action of heat.

Digestion of the fibres by a mucoproteinase (E 170) which was kindly given us by Professor Banga, destroyed (in 1 per cent solution at 38° C) the helical bands and transparent glassy fibrils remained. The latter showed thermic contraction. If, however, the digestion continued, they broke under a small load.

Digestion with hyaluronidase on the contrary did not specifically influence the helical bands (Garrett and Flory, 1956; Gortner, 1954; Hall *et al.*, 1952; Knox *et al.*, 1956; Lansing *et al.*, 1952; Lansing, 1955).

These experimental findings afford a possible explanation of the nature of the helical structure. With the onset of thermic contractions, helical bands are broken but so long as some remain the fibre is still contractile. Once they have disappeared and the fibre has reached the transparent stage, it becomes elastic. This seems to mean that the helical substance represents those 'bonds or cross-links' which in the natural fibre inhibit the contraction of the elastic proteins.

This latter substance, which constitutes the transparent fibre and which we call here elastic protein, seems to be identical with metacollagen (Banga *et al.*, 1954, 1955) and with elacin of former authors.

It is known that weak acetic acid dissolves mucopolysaccharides from the collagen fibre (Nageotte, 1927; Loewen, 1955a, b). Thus it is probable that the helical structure is a mucopolysaccharide, cross-linked with the elastic protein fibrils, the metacollagen. The mucopolysaccharide prevents the action of elastase on the natural collagen fibre. It has been shown by Banga that only a small quantity of mucopolysaccharide is dissolved during the contraction and another larger part when the contracted fibre becomes transparent. This would mean that heat first destroys only certain cross-linkages which inhibit in the natural fibre the contraction of the elastic protein fibres. If heat, acid or proteinase is allowed to act for a longer time, more cross-links are broken, and the fibre becomes completely elastic. Thus it seems that our observations identify mucopolysaccharide or rather mucoprotein as the substance of the helical structure cross-linked with an elastic protein.

Certain observations on the effect of thermic contraction on these tendon fibres show that inorganic ions also play a part. If tendon fibres are bathed in distilled water for several hours or days and then heated, the thermic contraction becomes smaller and the induction time much longer. If one transfers such fibres to a 0.9 per cent NaCl solution and then heats them to 62° C, the thermic contraction immediately becomes normal in strength and time. While Na^{++} ions have this action, Ca^{++} ions have no such influence in physiological concentrations. We also did not succeed in changing in a reversible way the thermic contraction after treatment of the tendons with a calcium ion-exchanger. Possibly calcium is more important for the binding with elastin than with collagen.

Age changes in the rat-tail tendons cannot be readily shown by means of simple stretching (although it is true that old tissue is somewhat less extensible than that of young animals) (Gottschlich, 1893; Roy, 1880; Remington, 1945; Banfield, 1952). Nor is it possible to see microscopic differences. Electron microscopic studies have shown that the interfibrillar ground substance decreased in tendons of aged animals (Schmitt *et al.*, 1942; Schwarz and Dettmer, 1954; Rollhauser, 1950; van den Hooff, 1952; Bahr, 1951; Gross, 1952; Küntzel, 1941).

Remarkable changes can be shown in the thermic contraction of

FIG. 1
Helically packed tendon fibres from the rat's tail.

FIG. 2
Thermic contraction of a rat's tail tendon fibre.
(*a*) Fresh single tendon fibre at 20° C. in Ringer's solution.
(*b*)–(*f*) Consecutive stages of the same fibre by heating up to 62° C.

tendons of animals of different age. These can be described in the following way: With ageing the weight which can inhibit thermic contraction continuously increases, all other conditions being equal (Fig. 3). The following examples show that tendons of older animals can lift greater loads. This change appears as early as the third month. In our rat colony (Verzár, 1955) maturity is reached in the fifth month; we consider them fully grown at 9-10 months; only 50 per cent of the animals reach the age of $23\frac{1}{2}$ months and only a small percentage reaches the age of 33 and very few 41 months; thus 18-24-month-old animals are called old.

Fig. 3 shows the load necessary to prevent thermic contraction.

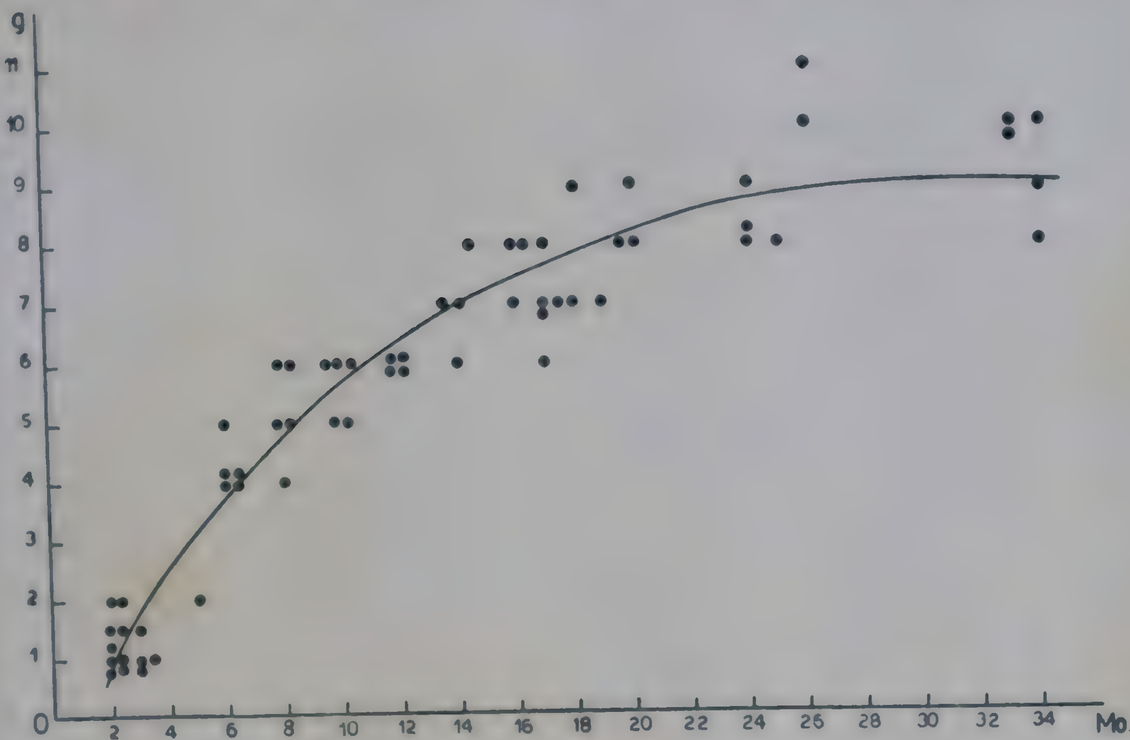

FIG. 3

Inhibition of thermic contraction by different weights in tendons of animals of different age.
Abscissa: Age in months.
Ordinate: Weight in grammes.

It is more practical for the purpose of comparison to compare the weights which can be lifted to 50 per cent of the maximal thermic contraction. These experiments were all done with the method of heating the tendon to a temperature of 66° C, above which no further contraction takes place.

It must be emphasized, contrary to the general description, that there does not exist a characteristic temperature at which thermic contraction occurs. The temperature depends on the load, all other

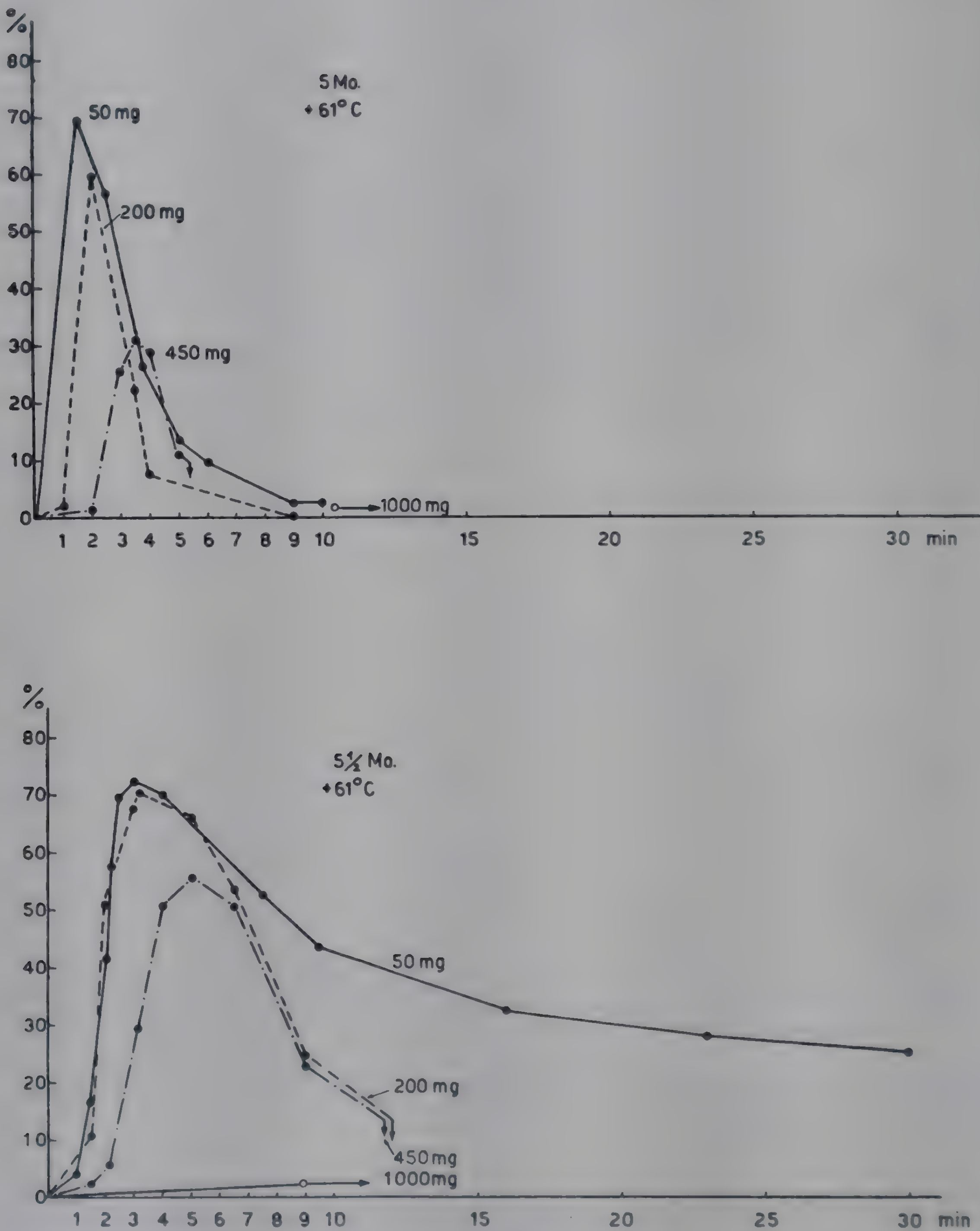

FIG. 4

Thermic contractions with different weights at 61° C in 5-, 5½-, 26- and 28-month-old rats' tail tendon fibres.
Abscissa: Minutes.
Ordinate: Percentage of contraction of original length of fibre.
Note the long induction times with larger weights especially in the tendons of the old animals.

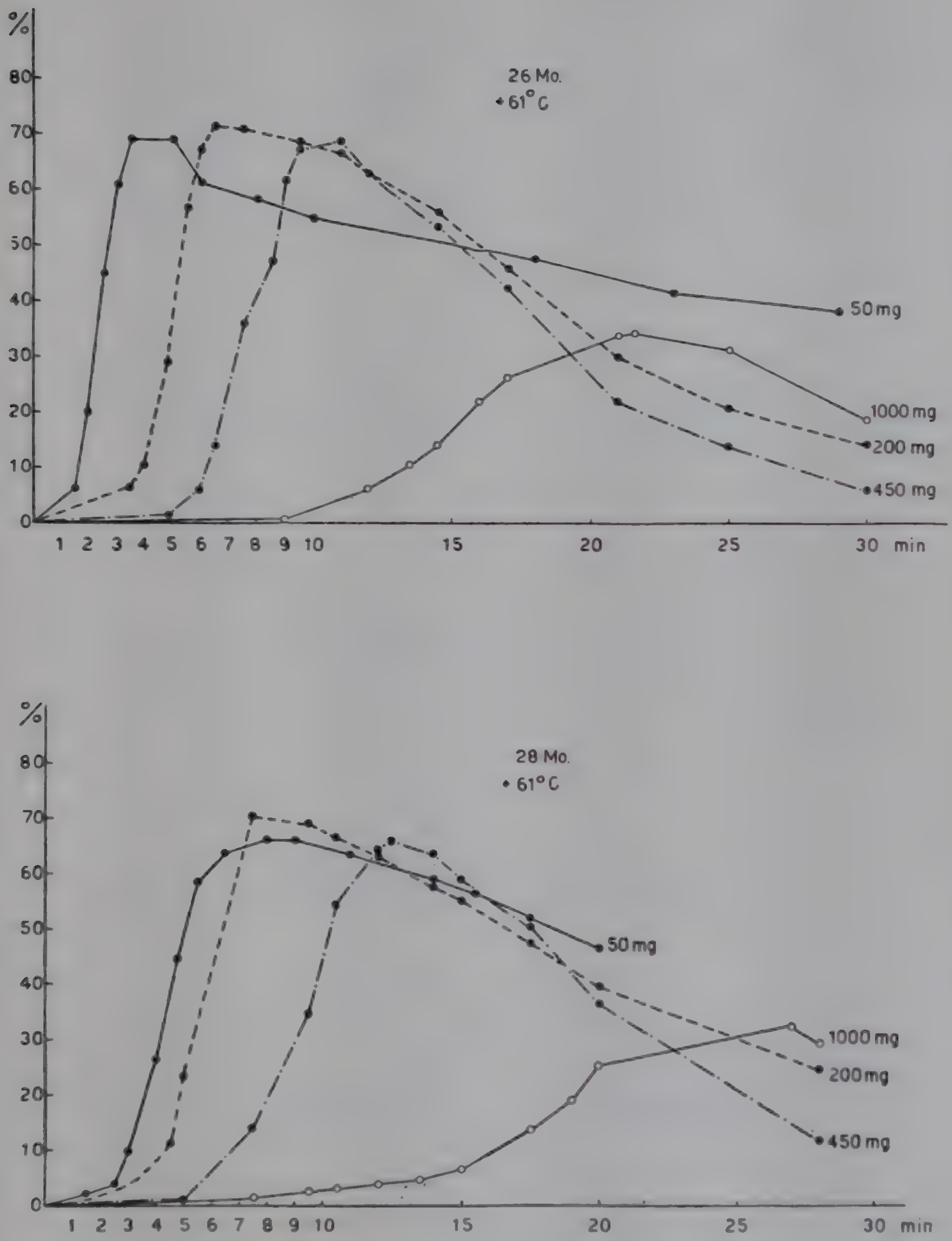

FIG. 4
Thermic contractions with different weights at 61° C in 5-, 5½- 26-, and 28-month-old rats' tail tendon fibres.
Abscissa: Minutes.
Ordinate: Percentage of contraction of original length of fibre.
Note the long induction times with larger weights especially in the tendons of the old animals.

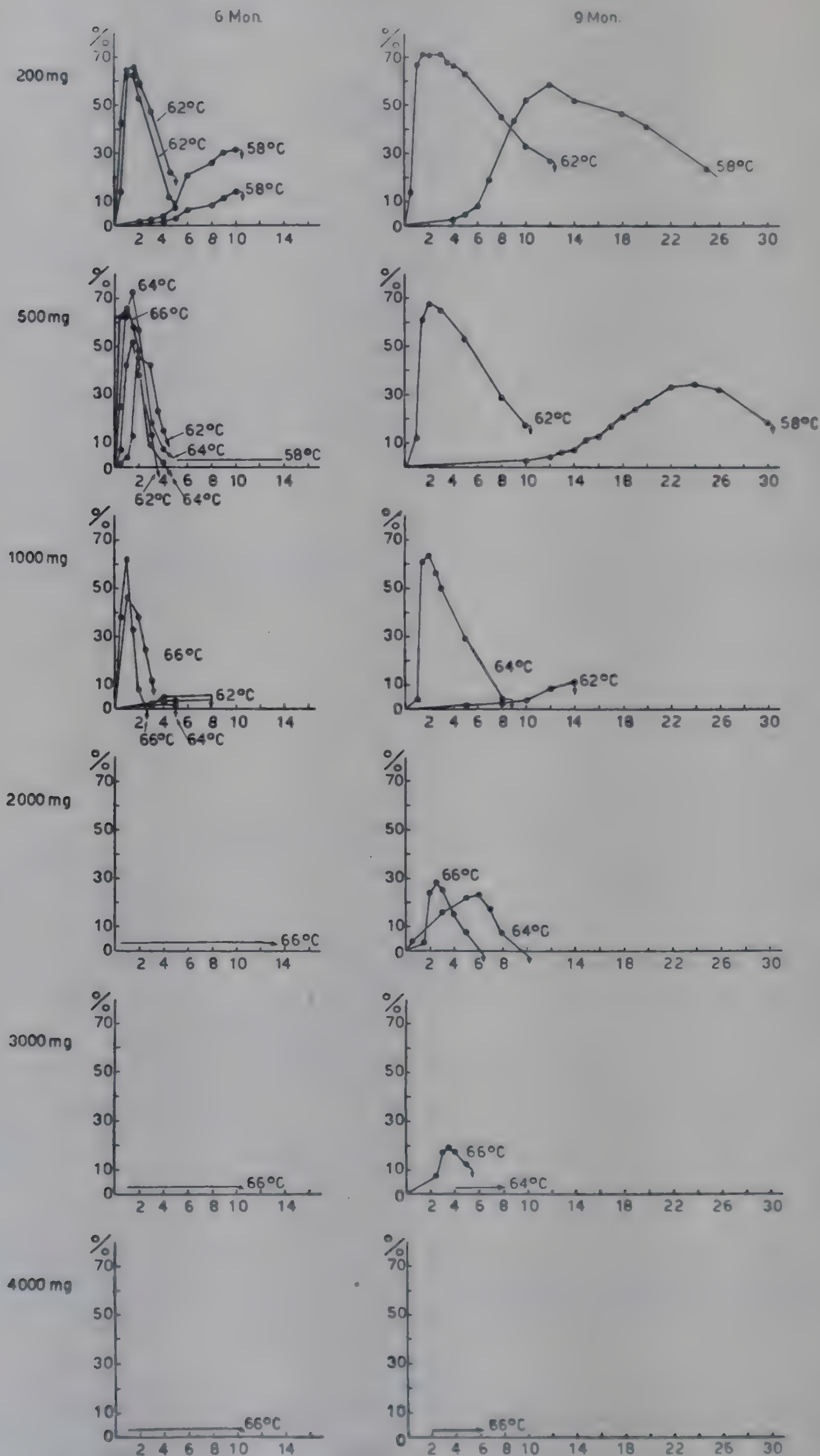

Fig. 5

Thermic contraction of 6-, 9- and 22-month-old rats' tail tendon fibres at different temperatures and weights.

Abscissa: Minutes.

Ordinate: Percentage of contraction of original length of fibre.

Note: Lower temperatures have longer induction time; larger weights need higher temperature; older animals' tendons lift larger weights at higher temperatures.

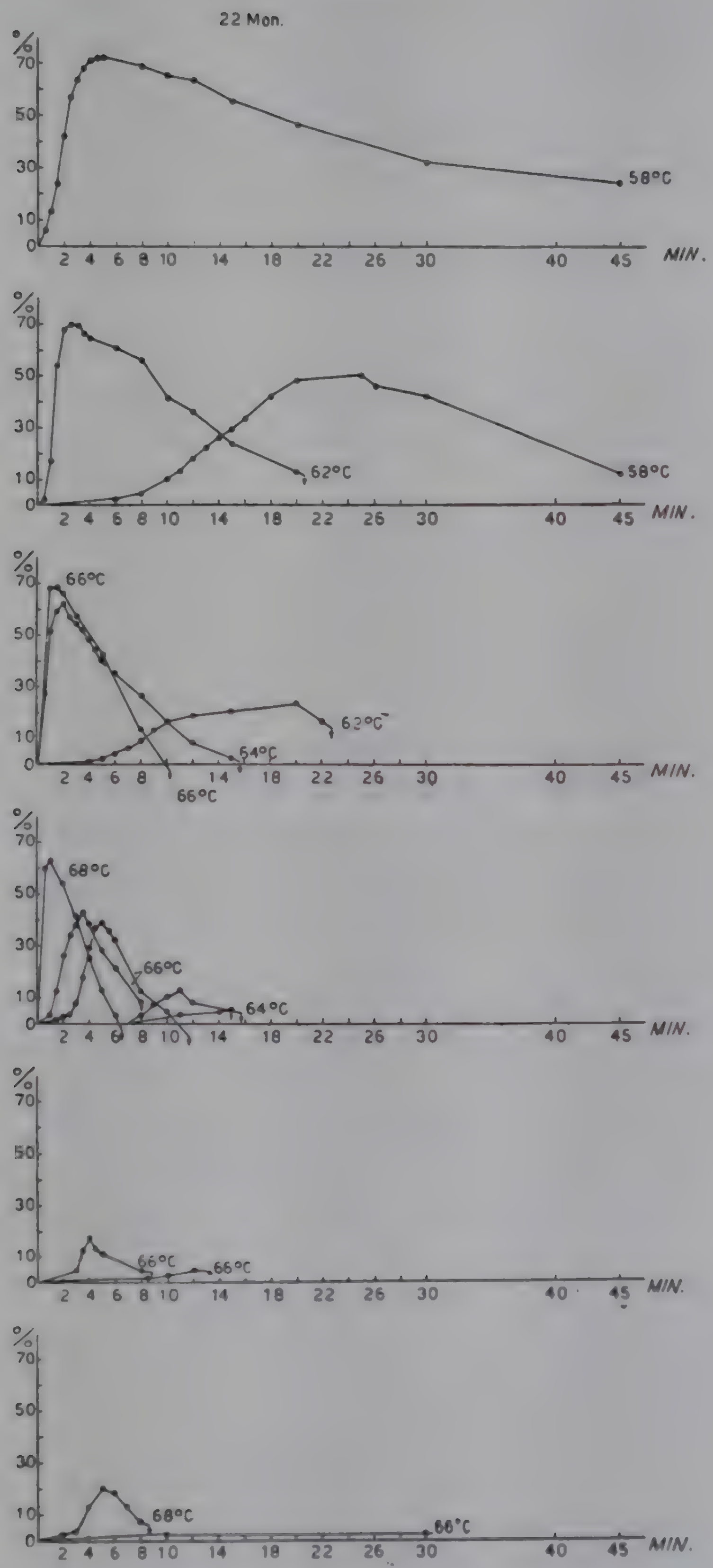

FIG. 5

Thermic contraction of 6-, 9- and 22-month-old rats' tail tendon fibres at different temperatures and weights.

Abscissa: Minutes.

Ordinate: Percentage of contraction of original length of fibre.

Note: Lower temperatures have longer induction time; larger weights need higher temperature; older animals' tendons lift larger weights at higher temperatures.

conditions being identical. Tendons of the same animal show thermic contraction with a larger load at a higher temperature. With a small load the induction time is shorter and the height of the contraction is higher. With only 100 mg. load already at 54° C, after a very long induction time, a small contraction may occur (Fig. 4).

This is also to be applied to different ages. The tendons of old animals contract with greater loads and do so at temperatures at which the tendons of younger animals do not contract (Fig. 5). If the comparison is made with small equal loads, young animals' tendons may not show any thermic contraction while old animals show it at 54° C.

Furthermore, as has already been said above, the so-called reversibility of thermic contraction is only the result of the elasticity of the tendon fibre which appears after thermic contraction. There are again remarkable differences between this so-called reversibility in young and old animals' tendons. Small weights reverse the contraction much less and more slowly in the old than in the young fibre (Fig. 5). A weight of 200 mg. may completely and quickly reverse thermic contraction in a young animal, while the tendon of an old animal may remain for a long time contracted with such a small weight. It will immediately be elongated, however, if the contraction is made with a weight of 1000-2000 mg.

These changes in the thermic contraction are such exact signs of the age that it becomes possible to judge the age of an animal on the basis of thermic contraction of a tendon fibre which was extracted under narcosis from the animal's tail. There are differences between the fifth and the tenth month, and much greater differences between the tenth and eighteenth and finally the thirtieth month of the animal's life.

After having described these physical differences in the behaviour of single tendon fibres in animals of different age, we shall consider the physico-chemical explanation.

To quote Gustavson (1956, p. 202): 'The contraction of the collagen fibre is generally considered to result from the weakening or dislocation of the interchain cross-links of the collagen fibrils. Heat interferes with cross-links.'

If the supposition which we made from the comparison of the optical, mechanical and chemical facts is right, then the optically visible helical bands represent mucoproteins. These inhibit by

cross-linkages the shortening of the contractile protein fibres. The destruction of the helical band is the destruction of cross-linkages and the elastic protein then contracts.

This by itself could not explain how the fibre of old animals can lift ten times greater weights than the tendon fibre of the young. One has to assume that more cross-linkages are present in the single protein chains of the old animals' tendon fibre. Since we have seen that the helical structure goes through the whole diameter of the fibre, it may be that cross-linkages with mucoproteins are responsible for these interfibrillar cross-linkages. But even then it seems that after the destruction of the whole helical structure, in the transparent stage, when the fibre is completely elastic, still more cross-linkages are present in the old fibre than in the young one, as judged by the lesser elasticity of the former.

I have to thank Professor Werner Kuhn for a discussion of these problems (1956). On the basis of a comparison with his studies on artificial systems, especially on polyacrylic acid with polyvinyl alcohol, he comes to the following analysis: 'In the case of aged rats a considerable part of the collagen must be present as almost completely stretched molecular chains, these being cross-linked to form a three-dimensional network. In young individuals practically the same molecular chains, possibly with the same degree of orientation, must be present, but without cross-linking. At room temperature any changes of the shape of the molecular chains are impossible due to the van der Waals forces (similarity to stretched frozen rubber). Elevation of temperature gives the chain molecules a certain amount of freedom of Brownian motion. In this state the difference between young and old collagen shows itself. In the case of young individuals (lacking cross-linking) no or only weak contractile force of the macroscopic sample is observed, the individual chain molecules reassuming at 62° C, by Brownian motion, each one by itself, its most probable shape in the medium. In the case of the old individual (cross-linking of the chains), the chain molecules transfer the change of their shape to the macroscopic sample giving rise to a contractile force of the latter. At still higher temperatures, the cross-linking collapses in all cases . . . Thus the differences in the elasto-mechanical behaviour of young and old collagen seems to be based on the cross-linking of the stretched collagen molecules being more pronounced and more stable in old than in young individuals.'

The idea that an increase of cross-links appears with age and is

mainly responsible for ageing, was discussed by Bjorksten in 1951 on facts of literature. While he collected only indirect proofs, the possibility that ageing in general might mean an increase of cross-linkages between filamentous proteins was discussed in an interesting way. King (1946) has also discussed the decrease of the elasticity of arteries in old age with application of the statistical mechanical theory of elasticity of high polymers. He came to the conclusion that the changes of elasticity are the result of the degree of cross-linkage of protein filaments which increases with age.

Wood in 1954, quoting the former work, wrote: 'It seems possible that if ageing is accompanied by cross-linking . . . a study of the mechanical properties of the tissue might help to elucidate the process.' Without having known about these earlier ideas, it seems to me that our work has produced mechanical proof for the increase of cross-linkages of collagen fibres with age.

GROUP DISCUSSION

Dr. Bear said that Küntzel found that the waviness of fibrils in tendon could be observed less clearly if the tissue had dried, and wondered if Dr. Verzár had studied the effect of drying.

Dr. Verzár replied that dried fibres do not show the helical structure. This appears when the fibres are immersed in water or Ringer's solution.

Dr. Bowes had observed similar helical banding on a larger scale when tendons were immersed in dilute acetic acid. As the acid penetrated and the fibre began to swell opaque bands appeared but later disappeared as the tendon became translucent.

Dr. Gustavson said that in 1948, Highberger found that formaldehyde tanning of collagen caused a slight decrease in tensile strength and concluded that no cross linkage had been introduced by the tanning. Dr. Gustavson expressed the opinion that cross linkage had nothing to do with strength but that thermal shrinkage is a function of cross linkage and agreed with Dr. Verzár's explanation of old age changes. He further referred to the work of Stucke who found the strength of tendons to increase up to the age of 20–30 years and then to decrease sharply. This is in harmony with the view that cross linking of the collagen chains has nothing to do with the tensile strength of the structure.

Dr. Reed asked if the helical structures observed by Dr. Verzár might be due to mechanical distortion of the fibre during its removal from the tail. Dr. Verzár said he had checked this point and found it was not so.

Dr. Fitton Jackson wondered if the observed phenomena might be

caused by diffraction effects due to the optical method employed and asked if similar phenomena had been observed in polarized light. She said that form birefringence of the fibrils is apparent during contraction which confirms the presence of numerous small fibrils parallel to the axes of the collagen bundle. She suggested that the large banded appearance might result from the flattening of a wavy fibre bundle. DR. BEAR said that it was important to remember that the tendon is a tissue (hence contains other fibrous components of various orientations) before drawing conclusions from observations such as Dr. Verzár had shown. He mentioned the possibility of mechanical effects occurring during removal of the tendon from the tail.

DR. GILLMAN said that it was possible to observe striations in rat-tail tendon *in situ* before it had actually been removed from the tail. In relation to the suggestion by Dr. Verzár that elastin-like fibres are formed during contraction and relaxation, DR. HALL asked if any evidence had been obtained of material being extracted from the fibres. DR. VERZÁR said he had done no work on this.

In reply to a question from Dr. Hall, DR. VERZÁR said that treatment with Banga's mucoproteinase gave the same results as treatment with low concentrations of acetic acid.

THE COMPOSITION OF MAMMALIAN ELASTIN

S. M. PARTRIDGE, H. F. DAVIS AND G. S. ADAIR

The work described in this paper has been carried out with a purified preparation of elastin powder. This substance is regarded as a chemically defined fibrous protein – it is believed to be in a state of substantial purity and to represent, as such, an integral component of the fibres of elastic tissue.

The yellow elastic tissue from *ligamentum nuchae* or *aorta* of cattle is known to contain, in addition to elastin, considerable amounts of collagen and smaller amounts of other proteins, mucoids and mucopolysaccharides including chondroitin sulphate. These are associated more or less intimately with the fibrous elements of pure elastin, which are themselves largely responsible for the characteristic physical properties of the tissue.

A procedure for the isolation of the protein 'elastin' must ensure complete removal of the other tissue components but at the same time it must not result in chemical damage to the elastin elements. It has been shown (Partridge and Davis, 1950), that, with many proteins, treatment with 2 per cent acetic acid solution at 100° for several hours results in the hydrolytic cleavage of peptide bonds at specific points in the chain. Similarly the work of Courts (1954) showed that on heating a sample of gelatin at 75° for 24 hours at pH 3.0, free α-amino groups were liberated and the mean molecular weight of the sample fell from 58,000 to 7000. Boiling with dilute acetic acid as a step in the purification of elastin was avoided therefore; instead a procedure based on repeated extraction with water in an autoclave at 120° was adopted. In order to ensure the complete removal of collagen and mucopolysaccharide, the dried powder produced after the first course of extractions was finely ground in a hammer mill before treating for a second time in the autoclave. Microscopic examination showed that this intermediate fine grinding separated any fibre bundles and thus exposed fresh surfaces to the action of boiling water. The final product, after again drying and grinding, consisted essentially of morphologically intact elastin fibres, which had the same staining properties as those to be seen in

tissue preparations. Comparison of the analytical data and physical properties shows that this product is closely similar to the elastin preparation described by Stein and Miller (1938).

The elastin preparation is not soluble in boiling 40 per cent urea solution even after 24 hours' treatment, but it should be noted that under these conditions urea solutions develop a strongly alkaline reaction, and very prolonged treatment with boiling urea results in partial or complete dissolution (cf. Hall, 1951) which may be caused by alkaline hydrolysis of peptide bonds.

The purified preparation of elastin from *ligamentum nuchae* was a free-flowing cream-coloured powder, which under the microscope appeared as short, smooth, rod-like fibres of almost circular cross-section. The fibres appeared to be remarkably uniform in thickness; measurement of 50 fibres gave a mean value of 6.5 μ for the diameter with a variation from 3.6 μ to 9.9 μ, whilst the lengths were of the order of 0.15–0.50 mm.

The fibres forming the dried powder from *ligamentum nuchae* were much thicker than the elastin fibres to be seen in loose connective tissue. These latter are observed as long thin threads, branching in many directions and staining strongly with orcein- or resorcin-fuchsin. Histological preparations of the fibres from *ligamentum nuchae* were made by embedding some of the powder in gelatin and fixing in neutral formalin solution; alongside this a number of preparations were made from abdominal areolar tissue from freshly killed rat and these were fixed with the same solution. Sections were then cut and stained both by Verhoeff's and Weigert-French's procedures for elastin. The sections were finally treated with the counterstains of van Gieson and Mallory.

The fibres from the powder preparation showed the same staining properties as the elastin fibres in the areolar tissue sections; the collagen and ground substance in the latter preparations did not retain the orcein-fuchsin stain but were well demonstrated by the counterstains.

SUMMARY OF EXPERIMENTAL WORK

Since the greater part of the experimental work on which this paper is based has already been recorded (Partridge, Davis and Adair, 1955; Partridge and Davis, 1955) it seems sufficient to give the main results here in summary form.

Partial Hydrolysis of Elastin from Ligamentum nuchae of Cattle

Treatment with boiling 0.25 M oxalic acid results in the complete dissolution of the fibrous protein and yields the mixture of soluble proteins which is studied in this paper. Six successive extractions, each for a period of one hour, were sufficient to bring dried and powdered elastin, from *ligamentum nuchae* of cattle, completely into solution. Removal of oxalic acid from the mixed extracts by dialysis through cellophane resulted in the loss of about 5 per cent of the nitrogen as small peptides.

Properties of the Soluble Protein

The non-diffusible protein was soluble in water at temperatures below 25° C to give a pale yellow mobile solution. On raising the temperature of a solution in dilute buffer at pH 4-6 a precipitate consisting of liquid droplets separated. The droplets showed no birefringence under crossed nicols and immediately dissolved again on reducing the temperature. Fig. 1 shows the temperature at which the phase separation first appeared when the pH of the solution was varied at constant ionic strength. On centrifuging at 37° C the droplets coalesced to form a lower layer of viscous liquid, and refractive index measurements showed the two liquid phases to consist of aqueous protein solutions of different concentration.

Electrophoresis Experiments

When dialysed against buffer mixtures of ionic strength 0.20 the protein showed a single symmetrical peak in the electrophoresis apparatus at all pH values in the range pH 2-9. If the ionic strength was reduced to 0.02 the protein showed a single peak in the range 4.7-7.5 but below pH 4.7 a boundary anomaly was encountered which resulted in unsymmetrical peaks, particularly in the ascending limb. Fig. 2 shows the electrophoretic mobility of the protein at ionic strength 0.02 and 0.20 when the pH was varied in the range 2-9.

Measurement of Isoelectric Point

At ionic strength 0.2 the value for the isoelectric point from electrophoretic mobility measurements was 3.9 while that from membrane potential determinations was 4.0. On reducing the ionic strength to 0.02, there was a considerable change in the position of the isoelectric point; determinations in the dilute buffer gave 4.8

from electrophoresis measurements and 4.7 from membrane potentials.

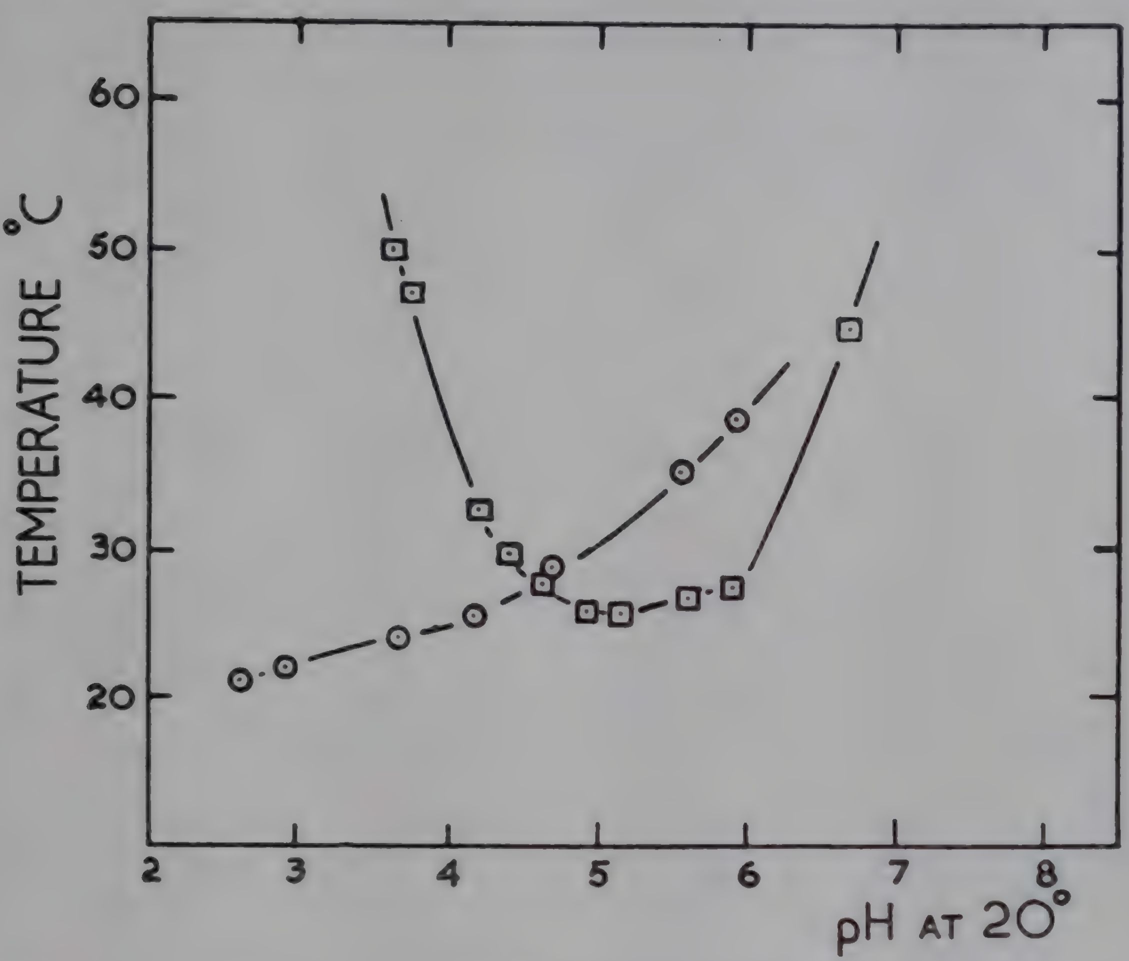

FIG. 1

Temperature of first appearance of phase separation in a solution of the mixed soluble proteins from elastin. Protein concentration 0.72 per cent (w/v). ⊡, acetate or phosphate buffer, ionic strength 0.01; ⊙, buffer of ionic strength 0.01 with addition of NaCl to a total ionic strength 0.10. The coacervate phase appeared on raising and disappeared on lowering the temperature.

Fractionation of the Soluble Protein

Fractionation by repeated precipitation of the liquid droplets which formed on raising the temperature or by use of a highly permeable collodion membrane resulted in the separation of two components with differing physical properties. One of these, which we called α, showed the characteristic property of reversible heat precipitation. Osmotic pressure determinations for this fraction gave mean molecular weights of 60,000 to 84,000 in different preparations. The second component, which we called β, gave no precipitate at any temperature up to 100° C and had a mean molecular weight of 6000 from osmotic pressure measurements.

It was at first thought that the substances α and β may represent inhomogeneity in elastin as it exists in the tissue, and a study of the course of the hydrolysis of powdered elastin by dilute oxalic acid appeared to support the view that the substance is a two-component system. Fig. 3 shows the amounts of α and β protein contained in five successive extracts, prepared by boiling a sample of elastin powder with M/4 oxalic acid for 1 hour periods. It will be seen that the

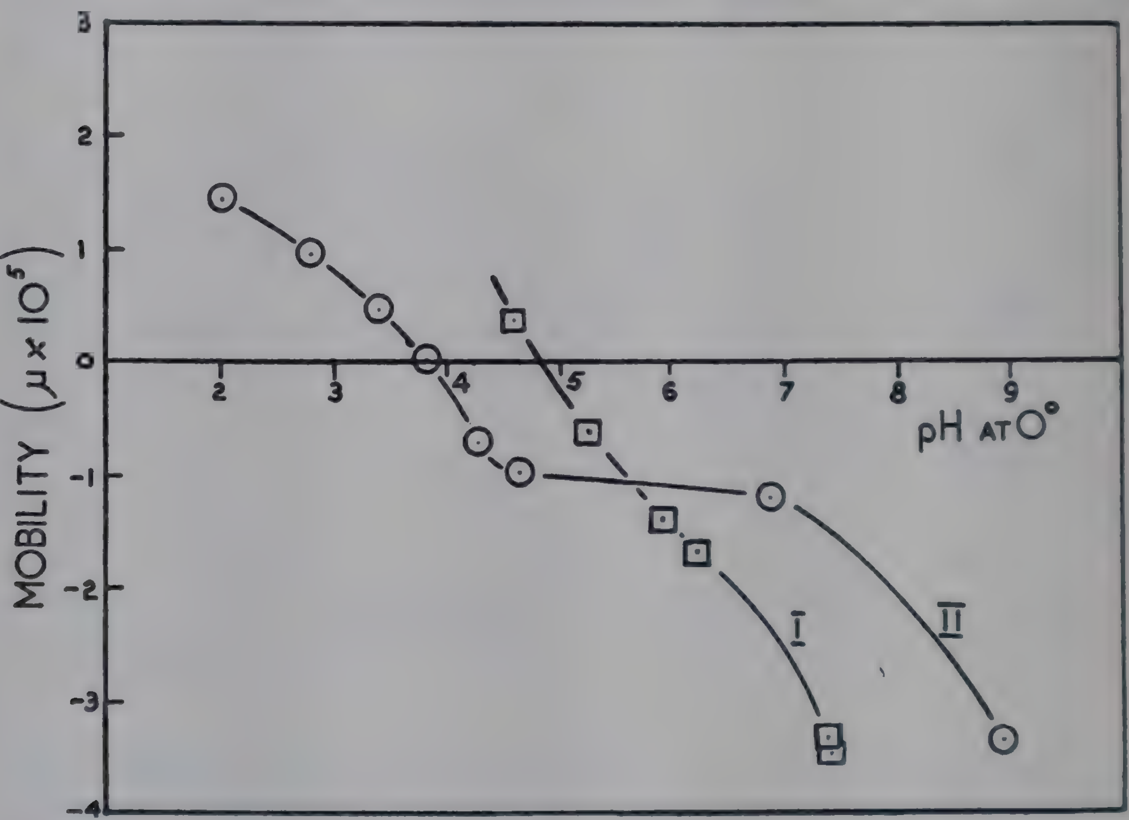

FIG. 2
Electrophoretic mobility of the mixed soluble proteins from elastin. ⊡, buffer of ionic strength 0.02; ⊙, buffer of ionic strength 0.02 with the addition of NaCl to total ionic strength 0.20.

early extracts are very rich in β-protein (M.W. 6000) while the later extracts are rich in α-protein (M.W. 60,000-85,000). This is the reverse of what would be expected if the protein β was a product of further hydrolysis of a soluble protein of higher molecular weight, and suggests that β arises from a more easily hydrolysed component in the original tissue.

Amino Acid Composition

The question remains as to whether, if there are two components in elastin, they are of different chemical composition or whether, on

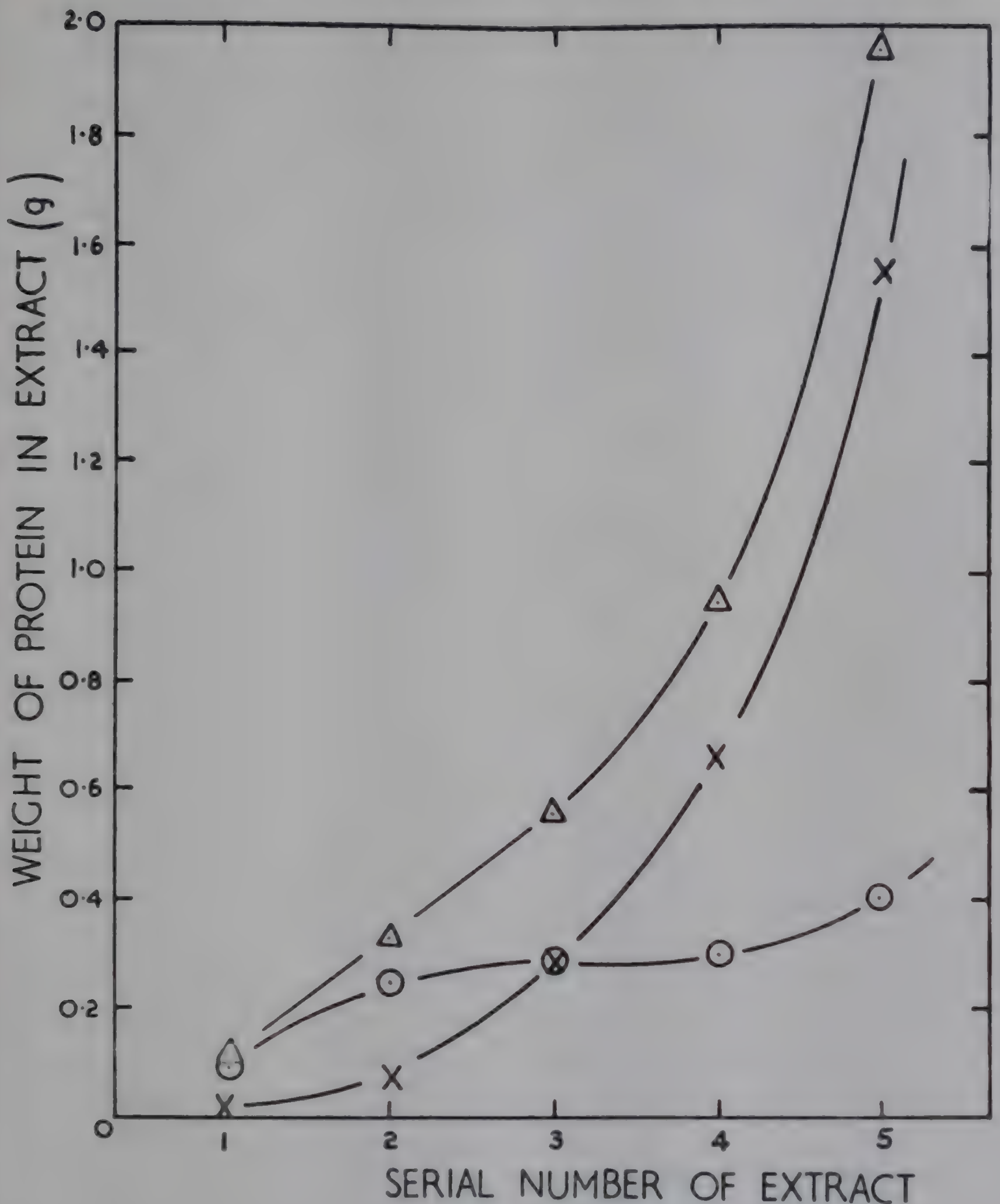

FIG. 3
Extraction of 5 gm. of elastin powder by heating with successive 50 ml. quantities of 0.25 M oxalic acid at 100° for 1 hour; △, gm. total protein dissolved in each extract; X, gm. α-protein released; ⊙, gm. β-protein released.

the other hand, they consist of basically the same kind of chain, but differ in their state of physical aggregation or degree of crystallinity. Accordingly, the amino-acid composition of the original elastin powder, the α-protein and the β-protein was determined by elution chromatography using ion exchange resins following the procedure of Moore and Stein. The results are given in Table I.

TABLE I

AMINO-ACID ANALYSES OF ELASTIN AND THE PROTEINS DERIVED FROM IT BY MILD ACID HYDROLYSIS

	gm. amino acid/100 gm. protein dry wt.				
	Elastin from ligamentum nuchae *prep.* 1	*prep.* 2	α-*Protein*	β-*Protein*	*Elastin from Aorta*
Glycine	26.7	27.0	23.2	29.0	26.2
Alanine	21.3	23.1	24.5	20.8	21.6
Leucine	9.0	8.9	8.3	8.1	9.5
Isoleucine	3.8	3.7	3.0	3.8	4.3
Valine	17.7	17.0	15.5	20.6	18.0
Serine	0.85	1.0	0.89	0.85	1.5
Threonine	1.1	1.2	1.1	0.98	1.7
Proline	13.5	12.8	12.3	13.8	14.5
Phenylalanine	6.2	6.2	—	4.6	6.2
Tyrosine	1.5	1.2	1.6	1.1	2.6
Methionine	Trace	Trace	Trace	Trace	0.53
Histidine	—	0.09	0.03	0.05	0.31
Arginine	1.3	1.2	1.2	0.93	1.9
Lysine	0.50	0.54	0.56	0.48	1.2
Aspartic acid	1.1	1.1	0.66	1.0	2.1
Glutamic acid	2.4	2.6	2.6	2.1	3.9
Hydroxyproline	1.6	1.6	1.5	—	1.7
Cystine	0.35	—	0.40	—	—

The analyses show that the amino-acid composition of the three proteins is substantially the same, and thus it appears that elastin powders, as usually prepared, may be regarded, as they have been in the past, as essentially homogeneous for the purpose of chemical investigations. Table I also includes the amino-acid analysis of elastin from bovine aorta prepared in exactly the same way as the preparations from *ligamentum nuchae.* The results, together with those from elementary analyses, indicate that the proteins prepared from either type of tissue are very similar chemically. Comparable data have been given by Neuman (1949) for elastin from *ligamentum nuchae* and by Lansing *et al.* (1951) for elastin from old and young aorta. It both cases the analyses were based on microbiological assay and the data indicates greater differences in amino-acid composition than were observed in this work.

Titration Experiments

Turning now to the structure of the soluble proteins, previous work has shown that a considerable degree of hydrolysis of peptide links occurs during the extraction with M/4 oxalic acid. The hydro-

lytic procedure releases aspartic acid and probably glutamic acid in a free state, and at the same time cleaves the protein chains at other positions where labile peptide linkages are to be found. This results in the production of new titratable α-amino and α-carboxyl groups. Titration curves determined by Mr. J. R. Bendall (Bendall, 1955) show the presence of many α-carboxyl and α-amino groups in both the soluble proteins. From the titration data it is concluded that during the treatment of the native elastin to give the α- and β-proteins, about 26 and about 31 moles of α-amino residues respectively are released in 10^5 gm. of protein, with the simultaneous release of corresponding numbers of α-carboxyl residues.

Estimation of Terminal Groups

Application of the fluorodinitrobenzene technique of Sanger gave the results shown in Table II.

TABLE II

NUMBER OF MOLES OF N-TERMINAL RESIDUES IN 100,000 GM. PROTEIN

	Elastin	*α-Protein*	*β-Protein*
Leucine } Isoleucine }	Trace	2.6	2.9
Valine	Trace	4.0	6.6
Alanine	0.08	8.6	11.0
Glycine	0.12	8.8	12.0
Serine	0.05	Nil	Nil
Aspartic acid	0.04	0.4	Trace
Total	0.29	24.4	32.5

The small number of N-terminal residues in elastin are probably not significant since they may be due to traces of collagen breakdown products remaining in the elastin preparation after removal of collagen by autoclaving.

It is significant that the α- and the β-protein contain almost equal amounts of N-terminal residues in unit weight, in spite of the very great difference in their molecular weight. This is taken to mean that they both consist of polypeptide chains of roughly equal length, and that the larger protein molecule must be built up by the binding together of a number of such unit polypeptide chains.

DISCUSSION

The discovery that the product of mild acid hydrolysis of elastin contained two proteins with markedly different physical properties led to the postulation of three alternative views of the macromolecular structure of the protein comprising the elementary elastin fibre.

(1) The protein is composed of two components of differing chemical composition.

(2) The protein is composed of two components which consist basically of the same kind of polypeptide chain, but differ as a result of differences in structural configuration.

(3) The protein is homogeneous, and the production of two degradation products of different molecular weight is due to the loosening of a network by removal of parts of it as low-molecular material.

A clear decision on alternative (1) has been obtained by determining the amino-acid composition of the original elastin powder, the α-protein and the β-protein. The results given in Table I show that the amino-acid composition of the three proteins is substantially the same, and thus it appears that elastin powder as prepared in this work may be regarded as essentially homogeneous for the purpose of purely chemical investigations.

The amino-acid composition of aorta elastin, though very similar to that from *ligamentum nuchae*, shows differences in respect of certain amino acids which are outside the range of probable experimental error. These differences may arise through residual contamination of the 'purified' preparations by collagen or degradation products of collagen. Removal of the last traces of collagen from preparations of elastin powder appears to be a slow and difficult process, and inspection of the data in Table I shows that the differences in the analytical figures for the aorta elastin could largely be explained by assuming a certain degree of contamination by a protein of collagenous origin. The difficulty of purifying elastin fibres becomes understandable in the light of observations reported by Dempsey and Lansing (1954) who state that when preparations of elastin purified with hot NaOH solutions are treated with elastase, collagen fibrils are liberated apparently from the internal structure of the elastin

fibre. The liberated collagen fibrils can easily be identified by examination with the electron microscope, since they show the characteristic cross-banded structure with major periodicity at 640 Å. According to Dempsey and Lansing the collagenous materials appear to be embedded in the elastic substance and may thus be protected from the solubilizing effects of the reagents designed to destroy them.

As regards the molecular structure of the two soluble degradation products derived from the elastin of *ligamentum nuchae*, previous work has shown that a considerable degree of hydrolysis of peptide links occurs during the extraction with 0.25 M oxalic acid. The hydrolytic procedure releases aspartic and possibly glutamic acid in a free state, and at the same time cleaves the protein chains at other positions where labile peptide linkages are to be found (Adair *et al.*, 1951). Titration curves carried out on elastin and the α- and β-proteins show the presence of many α-carboxyl and α-amino groups in both the soluble proteins. From the curves the α- and β-proteins contain 26 α-amino equivalents and 30 α-amino equivalents per 100,000 gm. of protein respectively. This result accords rather well with the information obtained by application of the fluorodinitrobenzene technique of Sanger (1945) which is reported in Table II. The number of N-terminal amino-acid residues found per 100,000 gm. of protein for elastin, α-protein and β-protein was 0.29, 24.4 and 32.5 respectively. The relationship between chain length and molecular weight is brought out in Table III. The data in this table shows

TABLE III

MOLES OF N-TERMINAL RESIDUES IN AMOUNTS OF ELASTIN DERIVATIVES CORRESPONDING TO THE MEAN MOLECULAR WEIGHT DETERMINED OSMOTICALLY

	α-Protein (67,000 *gm.*)	*β-Protein* (5,500 *gm.*)
From titration curve	17.4	1.72
From FDNB determination	16.2	1.84

that the β-protein consists of molecules composed, on the average, of 2 chains each containing about 27 residues, the N-terminal groups of which are variable. The α-protein has, as a mean value, 17 such

chains containing an average of 35 residues each, the N-terminal residues being glycine, alanine, valine and leucine with a small proportion of aspartic-acid terminals.

As yet there is little indication as to the nature of the cross-links which hold the chains together. Since the α-protein is derived from native elastin by a process involving rupture of the peptide chains, it seems probable that the cross-links in the soluble protein are the same as those existing in the natural fibrous protein. In this case the cross-links must be rather stable to acid hydrolysis, and the α-protein may then represent a 'resistant core' derived from the more highly cross-linked sections of the elastin fibre.

The rubber-like elasticity of the hydrated elastin fibre suggests that it is a rather disordered structure composed of bundles of randomly contorted peptide chains lying generally parallel to the fibre axis. For the greater part of their length the peptide chains must be free to take up independent thermal motion; but to account for the insolubility and the swelling properties of the fibre, the presence of cross-links at rather wide intervals must be assumed.

It is possible that such cross-links could be established by the presence of crystalline regions containing polar groups in close apposition, but there appears to be little evidence for such a structure from published X-ray diffraction studies. However, since elastin, unlike collagen, is resistant to the action of prolonged autoclaving, and shows no tendency to dissolve either in hydrogen bond-breaking solvents such as urea or in organic solvents such as phenol or cresol, it seems more probable that the primary covalency is involved in the relatively small number of cross-links present.

The only type of covalent cross-link that has so far been proved to exist in proteins is the -S-S- bridge due to two cystine half-residues. In elastin, however, cystine represents not more than 0.4 per cent of the protein dry weight, and this content would not provide sufficient cross-links to account for the stability of the structure. In order to ascertain if cystine bridges are an important factor in stabilizing the protein, elastin powder was treated with performic acid under conditions which ensured the conversion of the whole of the cystine half-residues to cysteic acid, thus breaking all -S-S- bridges. The oxidized protein remained insoluble after prolonged autoclaving, and was also insoluble in neutral 40 per cent aqueous urea and 90 per cent phenol both at room temperature and 100°. It thus appears that -S-S- bridges have little effect on the

solubility properties of the protein, and other stable cross-links must be present.

Cross-linking at the ε-amino groups of lysine or the phenolic hydroxyl groups of tryosine appears to be eliminated since it is shown that these groups are available for reaction with fluorodinitrobenzene. The amino-acid analysis of elastin and the α- and β-proteins shows them to have a remarkably low content of polar side chains. Aspartic and glutamic acid together account for 3.5 per cent of the dry protein; serine and threonine account for 2 per cent and hydroxyproline 1.6 per cent. It is possible that ester links formed between the hydroxyamino acids and such of the dicarboxylic amino-acid residues as are not combined with ammonia may account for the stability of the structure, but no evidence for the presence of such bonds has as yet been obtained. However, it should not be forgotten that since only approximately 95 per cent of the protein dry weight has been accounted for by amino-acid analysis, an unidentified substance may be involved in cross-bond formation.

A further possibility remains that the peptide fabric itself is involved either in covalent cross-linking or in aggregation at the molecular level by the intertwining of helical chains. Many workers now agree that the α-helix is the basic chain configuration in α-polypeptides and the α-forms of fibrous proteins, and various model systems involving molecular aggregation with the production of 'super-helices' have been discussed by Crick (1952), Pauling and Corey (1953), Ramachandran (1956) and others. In the case of elastin, a highly ordered structure appears to be unlikely, but the production of a network by random aggregation of helical chains could be envisaged. Such a model (alternative (3) above) would conceivably explain both the elastic properties of the natural fibre and also the production from it of two types of soluble protein by hydrolytic cleavage of the chains; the high molecular α-protein would then be regarded as composed of fragments of the three dimensional network, and the β-protein as portions of unidimensional protofibrils. However, the possibility of inhomogeneity at a higher level of organization should not be forgotten, and the present results are not incompatible with a model consisting of two structural components at the fibril level (alternative 2).

Lansing *et al.* (1952), using the electron microscope, observed the progress of dissolution of elastic fibres from *ligamentum nuchae* by the action of the pancreatic elastase discovered in 1950 by Baló and

Banga. They concluded that the fibres (diameter about 5 μ) were built up from many minute threads of fairly uniform thickness. These were approximately 25 mμ. in diameter and appeared to be the elementary units of elastic fibre in *ligamentum nuchae*. Varying numbers of these threads were loosely twisted together to form four thicker fibrillar components each of about 1 μ in diameter. The whole structure seemed to be cemented together and coated with a matrix material which had physical and chemical properties nearly identical with those of the fibrillar substance (cf. Bahr, 1951). The kinetics of the process of dissolution by elastase was followed by observing the liberation of Nile blue sulphate from stained elastic fibres, and the results suggested that the matrix material passed into solution at a much greater rate than the fibrillar substance. This result conforms closely with our own observations on the rate of liberation of the α- and β-proteins by mild acid hydrolysis and suggests that the fibrillar substance may be identified as the source of the highly cross-linked or branched α-protein and the matrix material as source of the β-protein which contains not more than two peptide chains linked together.

Recently Bowen (1953) has published a study of a soluble protein derived from elastin by prolonged boiling with 40 per cent (w/v) urea (Hall, 1951). This protein shows reversible phase separation on warming, and in some of its other properties resembles the α-protein described here. Sedimentation and diffusion experiments carried out with the protein derived from urea treatment suggested that the material may exist in solution at pH 4 as a dynamic equilibrium between more than one molecular species, dilution favouring breakdown into small molecules. By extrapolation to zero concentration a value for the mean molecular weight at infinite dilution of 6870 was obtained, and Bowen suggests that this may mean identity with the β-protein produced by oxalic acid treatment (M.W., 6000). A reliable value for the maximum molecular weight approached at high concentration could not be given, but in Bowen's view a value of 84,000, corresponding with the α-protein, would be in reasonable accord with the data.

In our work with the α- and β-proteins we have not observed any tendency towards inter-conversion of the two proteins due to alterations in the protein concentration; this is well brought out in the curves relating the effect of protein concentration on osmotic pressure (Partridge, Davis and Adair, 1955). Strong solutions of the

β-protein can be stored for prolonged periods without production of any material showing reversible phase separation on warming, and similarly, solutions of the α-protein will still show a milky appearance on raising the temperature at dilutions of 0.05 per cent (w/v) or less. We have had no opportunity of repeating the experiments of Bowen using the product derived from oxalic acid treatment, but the present evidence suggests that the proteins are not identical. It should perhaps be pointed out that during the treatment with boiling urea, ammonia is produced and the solution becomes alkaline. Solubility in urea may be brought about by partial alkaline hydrolysis, and the alkaline conditions may cause the rupture of interchain linkages which are more stable under acid conditions. The difference in properties of the two types of soluble degradation product may thus be due mainly to the absence in the urea product of stable cross-bonds. Work is continuing in an attempt to obtain further information on this aspect of the problem.

Action of Elastase on Elastic Tissue and Purified Elastin

The state of combination of the polysaccharide in yellow connective tissue and its possible effects in stabilizing the protein structure has been discussed by several authors, but some confusion has arisen largely as a result of inadequate definition of the preparations of elastin used for the experiments. Hall, Reed and Tunbridge (1952) found that polysaccharide and sulphuric acid is intimately associated with protein in elastic tissue and noticed the liberation of both during dissolution of elastin preparations by elastase or other agents. As a result they suggested that pancreatic elastase is not a proteolytic enzyme, but rather a mucase. Banga and Baló (1953) and Banga (1953) discussed the liberation of reducing substances during the action of elastase and about the same time Hall (1953) showed that the elastase preparations commonly used are probably complex and that at least one component has mucolytic activity. Wood (1953) showed that elastic tissue (from ox *ligamentum nuchae*) from which collagen had been removed by treatment with boiling dilute acetic acid still contains a polysaccharide which is bound interfibrillarly. The polysaccharide can be removed in large part by extraction with alkaline 10 per cent (w/v) calcium chloride and appears to be similar to chondroitin sulphate. Prolonged treatment of strips of ox *ligamentum nuchae* with alkaline calcium chloride solution at 37° had no observable effect on the elastic properties of the fibres, and from

this and other evidence it was concluded that there is doubt as to whether the polysaccharide associated with elastin plays an important part in structural stability.

The results of the present work show that the elementary fibres comprising the bulk of the native ligament consist of a protein which contains not more than 0.3 per cent of carbohydrate and insignificant amounts of ester-bound sulphate. The protein may be regarded as chemically homogeneous but it is not excluded that individual peptide chains, comprising the protein, may constitute a 'family' of molecules in which replacement of certain amino acids, one by another, is permissible. Estimation of free α-amino groups liberated during the action of elastase on this protein (Partridge and Davis, 1955) shows that enzymic degradation involves the rupture of many peptide bonds, and thus at least one of the components of 'elastase' must be regarded as a proteolytic enzyme.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

GROUP DISCUSSION

In reply to Dr. Neuberger, Dr. Partridge said that a large part of the aspartic and glutamic residues in elastin is in the amide form. Dr. Neuberger said that in view of the low histidine content of the beta protein, the 6000 molecular weight particles of this fraction must be dissimilar. Dr. Partridge agreed and said that this might arise from random hydrolysis.

Dr. Neuberger found it hard to see how the polypeptide chains in the alpha fraction could be joined together covalently and suggested that some reversible association, such as occurs in insulin, might occur in the alpha fraction.

Dr. Partridge said that measurements of the variation of osmotic pressure with concentration did not indicate that he was dealing with an equilibrium mixture. The great insolubility of elastin in hydrogen-bond breaking agents indicated the presence of fairly strong bonds (he had not tried dichlor-acetic acid). Even when the small amount of cystine present was oxidized with performic acid, the protein remained insoluble. The presence of a small number of strong bonds would also account for the elasticity of the fibres.

In reply to Dr. Meyer, Dr. Partridge said that 5–8 per cent of the

nitrogen content of elastin had not been accounted for in the Moore and Stein analysis. It was possible that some undetected nitrogen-containing substance might be involved in cross linking. There was no phosphorus in elastin.

DR. JACKSON had observed a shift of the iso-electric point of gelatin from both citrate-soluble collagen and insoluble collagen, as the salt concentration of the solution was increased. In both cases the iso-ionic point was pH 9.3 and the iso-electric point at an ionic strength of 0.1 was pH 5.1.

DR. PARTRIDGE said that it must be due to firm binding of the chloride ion by these proteins.

DR. NEUBERGER observed that both gelatin and elastin have very little buffering capacity in the pH range 6-8, so that a small amount of bound chloride ion would cause an appreciable shift in iso-electric point.

DR. GRASSMANN replied to a remark made by Dr. Neuberger on his own work on the degradation of collagen and procollagen with phenyl iodoso acetate. Although the product from both materials had an average chain length (end group determination) of 20-30 amino-acid residues, the product from procollagen behaves as a low molecular weight material whereas the product from collagen behaves as a high molecular weight substance, and he suggested that some interchain linkage must be present in collagen.

In reply to Dr. Baló, DR. PARTRIDGE said that his elastin preparations did not contain fat but pointed out that any fats on the outside of the native fibres would have been removed during his purification procedure.

In reply to Dr. Consden, DR. PARTRIDGE said that his elastin preparation contained only 0.3 per cent carbohydrate. Though this might be involved in cross linkage, he thought it was probably a contaminant. DR. SNELLMAN said that if, as seemed likely, there was only a small amount of cross linkage, the molecule must be highly hydrated. He thought that some intermolecular forces, similar to those which bind together the polypeptide chains in some peptide hormones might occur in elastin. DR. PARTRIDGE thought that in view of the high concentration of non-polar side chains in elastin, a high degree of hydration would not be expected.

DR. HALL said that study of the attack of elastase on elastin fibres by means of the electron microscope showed that an inner mass of fibrils was enclosed by an outer coating and suggested that Dr. Partridge's material might be composed only of the inner fibrils. He said that any lipoprotein or lipo-polysaccharide associated with the outer coating might have been removed during purification.

DR. PARTRIDGE said that the appearance of the fibres suggested that their essential structure had not been damaged by the purification procedures used.

CHEMICAL AND ENZYMATIC STUDIES ON ELASTIN

D. A. HALL[1]

Chemical studies on the structure of elastin have developed far more slowly than corresponding studies on the other fibrous component of connective tissue – collagen.

Ten years ago although characterization of elastin by histological means was commonplace (Unna, 1896), few facts were known regarding its chemical structure and these were confined to an incomplete amino-acid analysis, for example Stein and Miller, 1938. Since then electron microscopical (Wolpers, 1944; Gross, 1949 and Hall, Reed and Tunbridge, 1955) chemical (Bowes and Kenten, 1949, and Partridge and Davis, 1955) and enzymatic (Banga and Schuler, 1953; Hall, 1955 and Lansing, Rosenthal, Alex and Dempsey, 1955) examination of elastin has advanced rapidly. On the other hand the fundamental background for staining reactions has been studied mainly only in so far as the metachromatic reaction (Baló, Banga and Schuler, 1954) of the fibres is concerned and with the exception of one single observation little has been accomplished which could throw light on the empirical staining reactions which are specific for elastic tissue (Weiss, 1954).

The studies of the past decade have, moreover, been confused by numerous apparent contradictions between the reported results of various groups of workers. It was suggested in 1951 (Hall) that adequate characterization of elastin for any analytical procedure would entail the assessment of at least two properties with a view to proving the identity and purity of the preparation. Many of the discrepancies may well be attributed to the fact that whereas one group of workers may base the identity of their preparations on chemical analyses, others consider elastin to be pure if it consists of an histologically homogeneous species.

Having electron-microscopical, histological and chemical methods available, the Leeds group have aimed at a correlation of all three

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methods and have utilized them wherever possible for characterization of the starting material for analytical procedures.

One of the most controversial questions of elastin chemistry has been the evaluation of the role played by polysaccharide in the stabilization of the elastin molecule. Completely contradictory assessments of the evidence have been put forward by various groups of workers. Although as will be shown later, this divergence may be ascribed to actual differences in technique, semantic difficulties in the statement of the problem have added to the confusion. The concept of stabilization can be defined as the property which confers insolubility on elastin or the factor conferring resistance to enzymatic attack or even the factor conferring physical stability to the fibre itself. Although it is conceivable that the same groupings and linkages may stabilize the fibre in all three senses, it is equally probable that the structures necessary to confer insolubility, enzymatic inertness and physical strength are completely unrelated. It is, therefore, possible that the removal of a portion of the naturally occurring fibre could be accomplished without affecting the solubility of the fibre to any great extent and without completely altering its susceptibility to enzymatic attack, while markedly affecting the tensile properties of the structure. Without unlimited facilities it is virtually impossible to examine all these facets of the problem simultaneously and therefore caution has to be exercised in specifying the particular reaction under consideration when attempting to construct a model which will explain the stability of the structure and which at the same time satisfies the restrictions placed upon it by the particular definition of stability employed.

The hypothesis on which the author and his colleagues in Leeds have based their subsequent studies was first proposed in a paper by Hall, Reed and Tunbridge in 1952 in which they suggested that elastin could be considered as consisting of two phases, one amorphous and one fibrous, the former surrounding the latter and affording it protection against solubilization. This outer casing was described as containing a mucoprotein and its presence was regarded as the stabilizing factor for the fibre as a whole. Here the definition of stabilization employed was that of conferring insolubility on the fibre since it was believed that the enzymatic removal of the outer layer would bring about the spontaneous solution of the fibrous inner portion without the intervention of any further enzyme.

The existence of amorphous and fibrous phases was in agreement

with the model proposed by Meyer and Ferri (1936), which consisted of a steel spring maintained in a compressed state by a rubber band. It thus seemed possible that the mucoprotein portion of the fibre also acted as a stabilizer in the physical sense of the term. Later work by Wood (1954), however, showed that treatment of the elastin fibres of ox ligament with reagents likely to dissolve or degrade the polysaccharide had very little effect on its physical properties; in fact far less than could be observed when similar experiments were performed on whole ligament still containing collagen fibres.

A subsequent hypothesis was developed as a corollary (Hall and Gardiner, 1955) to the theory that elastin consisted of two components, namely that elastase was also dual in nature; one enzyme being specific for the inner fibrous protein portion and the other for the outer amorphous mucoprotein. This introduced the possibility that the mucopolysaccharide acted also as a stabilizer in the sense of a protective sheath against the action of one of the two enzymes. The anti-solubility and the protective aspects have received most attention in Leeds.

Recent work, which will be considered below, indicates how analysis of elastin and fractionation of elastase have paved a way for a more rational explanation of these phenomena on the basis of which an overall structural model of the elastin fibre can be built up.

PURIFICATION OF ELASTIN

Elastin is invariably present in tissues in association with collagen fibres and a mixture of the more soluble mucoproteins to which the generic term of ground substances has been applied. Any purification procedure must remove these two extraneous groups of material entirely before it can be assumed that the residue consists of a single entity to which the name elastin can be ascribed.

Various methods for the removal of collagen have been employed. In the main these employ the preliminary removal of ground substance by 5 per cent sodium chloride extraction followed either by acetic acid extraction (Gross, 1949; Hall, Reed and Tunbridge, 1952), sodium hydroxide extraction (Bowes and Kenten, 1949; Baló and Banga, 1950) or water (Partridge, Davis and Adair, 1955). Such various treatments produce material which is completely devoid of collagen. There is, however, a significant differ-

ence between the residues obtained. Elastic tissue may be refluxed in the presence of 2 per cent acetic acid for 5 to 6 days without any apparent change occurring in the fibres, either chemically or morphologically. Refluxing elastic tissue in the presence of 0.1 *N* sodium hydroxide, however, brings about the total dissolution of the tissue within six hours (Hall, 1955). It would appear likely that degradation begins after a far shorter period of time. The actual degree of degradation taking place in alkali is difficult to assess without a basis for comparison and it may be that acetic acid extraction also has the effect of liberating certain groupings of importance in the reaction with elastase, which would otherwise remain blocked. This possibility can be deduced from the figures in Table I which

TABLE I

EFFECT OF PURIFICATION ON THE REACTIVITY OF ELASTIN TO ELASTASE (17 HR. pH 8.7)

Elastin preparation	*Protein dissolved (per cent)*
Whole ligament powder (I)	19.3
Acetic acid treated I (II)	25.1
II Refluxed 1 hour with 0.1 *N* NaOH	51.2

reflect the amount of protein passing into solution under the action of elastase from preparations of ox *ligamentum nuchae* which have been treated with 2 per cent acetic acid at 120° C, under pressure for three hours, with that dissolved from similar material which has subsequently been boiled with 0.1 *N* sodium hydroxide for one hour. For comparison, figures are also included for similar studies on whole *ligamentum nuchae* still containing collagen and ground substance.

All three samples were ground to the same mesh size and the enzymic reaction was permitted to proceed under standard conditions. These conditions which have been those employed unless otherwise stated in all the experiments referred to in this paper are as follows:

50 mg. of elastin preparation suspended in 10 ml. of pH 8.7 borate buffer solution containing 250 µg. of elastase preparation, in

the case of solid enzyme. Where these are only available in solution, e.g. in more purified fractions, 1 ml. of solution containing between 60 and 70 μg. protein was added to 9 ml. of buffer. The mixture was then incubated at 37° for periods varying from 2 to 17 hours depending on the purity of the elastase preparation, and the protein dissolved estimated in the supernatant by means of the biuret reagent after the enzyme action had been stopped by immersion of the reaction mixture in boiling water for three minutes.

As can be seen, there is a marked increase in activity of the enzyme as one passes through the series of purification procedures. Electron microscope examination of the preparations obtained by treatment with acetic acid showed that all structurally discernible collagen had been removed. Chemical analyses (Hall, 1955) on the other hand, especially of preparations obtained from aorta, showed that there still remained an appreciable amount of material having an amino-acid analysis midway between that of elastin and collagen. This was not true of ox *ligamentum nuchae* preparations for which treatment with acetic acid appeared adequate to bring the amino-acid analysis into close conformity with classical values such as those obtained by Bowes and Kenten (1949) and more recently by Partridge *et al.* (1955). During treatment with alkali there was no marked variation in the amino-acid composition of the elastin from ligament fibres. Apparently the protein portion of the fibre remained unchanged in amino-acid composition until total dissolution occurred after six hours' treatment. There were, however, marked changes in the polysaccharide content of the residue after varying periods of boiling with alkali. During the first two hours' boiling the polysaccharide content fell rapidly although it never reached a value below 0.2 per cent, maintaining this figure until complete dissolution ensued.

It would normally be expected that even prolonged autoclaving with distilled water (Partridge *et al.*, 1955) would be a less drastic method of collagen removal than shorter periods of autoclaving with acetic acid. That this is not so, however, can be deduced from a comparison of the polysaccharide content of elastin preparations obtained by the method of Adair *et al.* and by the present author (Hall, 1955). The only explanation would appear to be that the acidic polysaccharides concerned are less soluble at pH 2 to 3 than at pH 6, and hence the fraction containing polysaccharide remains with the residue on extraction with acetic acid.

THE ENZYMATIC SUSCEPTIBILITY OF ELASTIN PREPARATIONS AFTER DIFFERENT PERIODS OF ALKALI TREATMENT

Partially purified elastase preparations show a striking degree of dissimilarity in their reactivity with elastin preparations that have received varying degrees of alkali pre-treatment (Hall, 1955) depending apparently on the precise nature of the preparative process employed. Fig. 1 shows the type of curve obtained, relating amount of protein taken into solution to the degree of pre-treatment of the substrate at a variety of enzyme concentrations, for an enzyme preparation obtained by the precipitation at 45 per cent ammonium sulphate concentration of a pH 4.5 sodium acetate buffer extract of defatted hog pancreas. Peak activity in terms of protein passing into solution occurs after alkali treatment amounting to between 30 and 60 minutes. Thereafter apparently the substrate of the enzyme concerned is either removed or altered in some way so that it is no longer susceptible to attack.

Subsequent experiments on the fractionation of such elastase preparations at various ammonium sulphate concentrations showed that the enzyme associated with this particular peak at intermediate periods of alkali treatment could be concentrated in a fraction precipitated at 20 per cent ammonium sulphate concentration (Hall, 1956).

A variety of other procedures which have been used for the fractionation of elastase have all afforded further evidence for the dual nature of the enzyme but have not been adequate for complete separation of the various components. Thus, precipitation with acetone, or adsorption and elution from alumina or from a column of powdered elastin, have provided enzyme fractions which were rich in one or other of the components, but which were not composed of one component alone. Complete separation of two active fractions has, however, been accomplished by zone electrophoresis on paper (Hall, 1956). The apparatus, which is an adaptation of that of Künkel and Tiselius (1951) is described in full elsewhere (Lloyd, Czerkawski and Hall, 1956) but the salient difference between it and other similar pieces of equipment lies in the use of two relatively thin perspex sheets forming the upper and lower surfaces of a pair of cooling chambers between which the paper can be clamped. It is thus possible to dissipate the heat generated very much more efficiently and because of this the current employed can be far higher

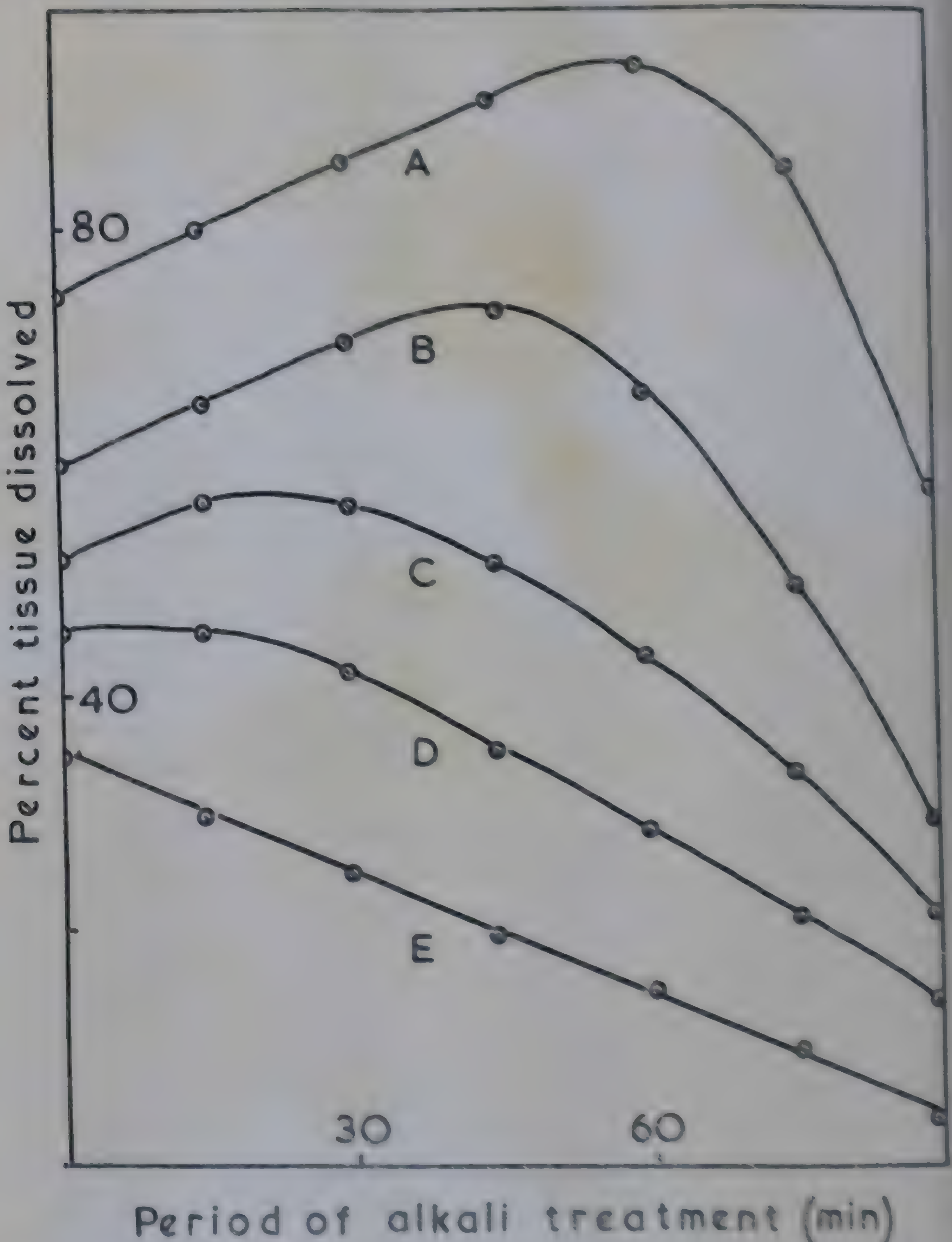

FIG. 1

The relationship between the percentage of tissue dissolved and the period of alkali pre-treatment at a series of differing enzyme concentrations. A, B, C, D and E refer respectively to enzyme concentrations of 100, 80, 60, 40 and 20 µg. per ml. of 0.1 M glycine buffer pH 8.7. 50 mg. of each substrate were incubated in 10 ml. of this solution at 37° C for 17 hours.

Dyeline Reproduction of the Central Part of an Electrophoretogram of Elastase

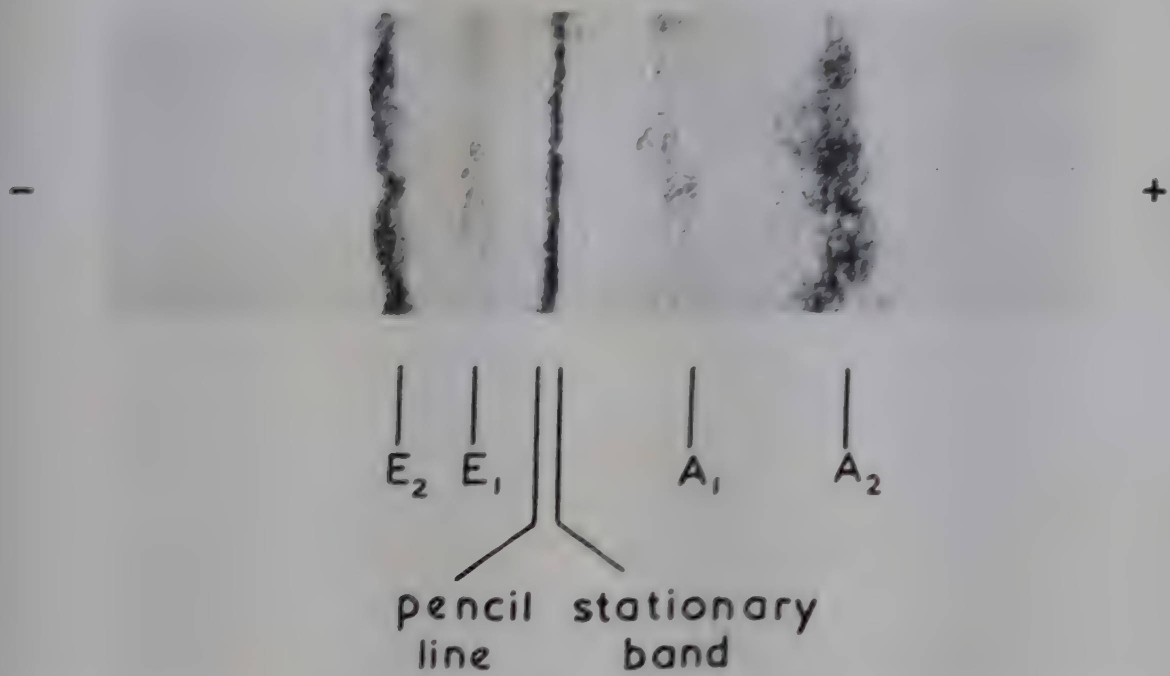

FIG. 2

The reproduction of the central five bands of an electrophoretogram of crude elastase preparation carried out in veronal buffer pH 8.6, $\mu = 0.1$ for $1\frac{1}{2}$ hours under a P.D. of 20 volts per cm.

The Components of Certain Soluble and Insoluble Fractions of Elastase

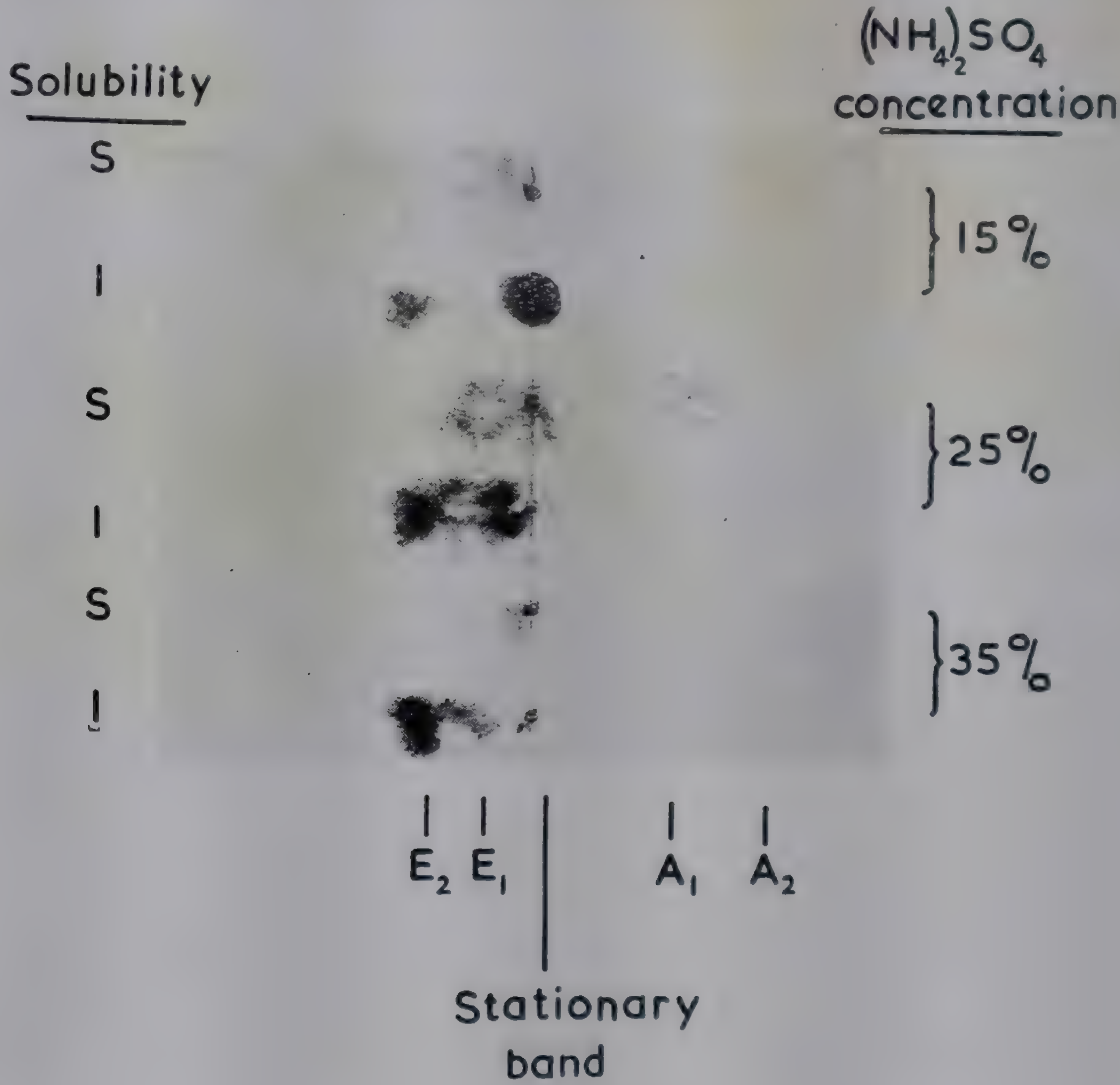

FIG. 3

An electrophoretogram carried out under similar conditions to those described in Fig. 2 showing the difference in composition of the soluble and insoluble (S and I) components of three fractions obtained by an ammonium sulphate precipitation between: 10 and 15 per cent, 20 and 25 per cent and 30 and 35 per cent ammonium sulphate concentration.

than that normally used. Adequate separation of serum proteins, for instance, is accomplished in $\mu = 0.1$ pH 8.6 veronal buffer in $1\frac{1}{2}$ to 2 hours, using an overall P.D. of 1000 V to give a maximum current of 40 mA.

Crude elastase preparations examined on this equipment show the presence of 10 or more bands completely separated from one another (Fig. 2). The bands are stained with Solvay Purple by the method of Jones and Michael (1950) after 30 min. heating at between 120° and 180° C. Prolonged heating is necessary because a number of the bands are very soluble and the staining procedure is carried out in aqueous solution. Unless they are firmly bound to the paper by heat denaturation they are lost during the application of the dye. These bands assume a grey-blue appearance as opposed to the pure blue tint of those which are sufficiently insoluble to be stained by drying at 100°. Of the 10 bands observed in crude elastase preparations, only one or two are completely removed by the various fractionation procedures. Ammonium sulphate precipitation, for instance, appears to affect the concentrations of various components so that between the two extremes of a series of ammonium sulphate precipitates, there is a gradual change in the intensity of the various bands on the electrophoretogram (Fig. 3). Only two of the bands appear to have any connection with the enzyme reaction. These are the first and second basic bands. Of these the least basic E_1 when tested alone appears inactive since it does not have the power to cause protein to pass into solution, which is the normal method of assessing activity. The second band E_2, however, does have this faculty. When they are both tested simultaneously, a 50 per cent increase in the measured activity of E_2 can be observed. The optimum ratio of E_1 to E_2 is 1 to 5, further additions of E_1 having no effect on the enhancement of activity of E_2. A number of experiments have been carried out utilizing a composite eluate containing both E_1 and E_2 in the proportions in which they were originally present in the impure enzyme and it has been shown that electrophoretic separation of these two factors from the rest of the elastase preparation effects the removal of at least one proteolytic factor. This can be demonstrated in the following manner:

Relatively impure elastase preparations bring about the initial appearance of protein in solution and this increases until the whole of the elastin preparation has been dissolved. On prolonged incubation, however, estimation of protein in solution shows a decrease indicat-

ing the presence and simultaneous action of a proteolytic enzyme degrading the proteins into small peptides and amino acids which are indeterminable by the biuret technique.

E (the composite solution of E_1 and E_2) is completely devoid of this enzyme since the concentration of protein in the elastolysate remains constant for periods up to 90 hours' incubation (Table II).

TABLE II

REMOVAL OF PROTEOLYTIC ENZYMES FROM ELASTASE BY ELECTROPHORESIS

Time of Incubation hour	*Biuret Readings Crude Elastase preparation*	*'E'*
0.5	0.5	3.8
1.0	2	10.4
2.0	8	23.8
4.5	16	29.8
12.0	27.5	29.6
16.0	29.2	29.6
24.0	28.1	29.7
40.0	25.0	28.9
90.0	22.0	29.1

Although elastase causes protein to pass into solution and there is no evidence to preclude completely the fission of peptide linkages, it would appear that solution is accomplished without recourse to generalized proteolytic action.

During the course of experiments on impure elastase preparations it had been shown, initially by Baló and Banga (1950) and later by the present author (Hall, 1953), that the shape of the pH activity curve was such as to indicate the presence of two distinct enzymes. In the papers of the Hungarian workers this was exemplified by the appearance of a curve with an optimum peak associated with a shoulder at a lower pH value. It could be shown (Hall, 1955) that under certain circumstances, particularly when the substrate had received intermediate periods of alkali pre-treatment the shoulder in the regions of a pH of 7.8 would transform into a peak, so that partially purified enzyme preparations tested against alkali treated substrates gave a double-humped pH activity curve with one peak at 7.8 and the other in the region of 8.7. On purification it was found that E_2 had a single peak but that due to changes brought about by the removal of extraneous material the peak had moved to

a lower pH value in the region of 8.4 to 8.5. When examined alone, E_1, as previously stated, showed no activity in terms of dissolved protein, but the addition of small quantities of E_1 to E_2 brought about the reappearance of the shoulder at the lower pH value (Fig. 4).

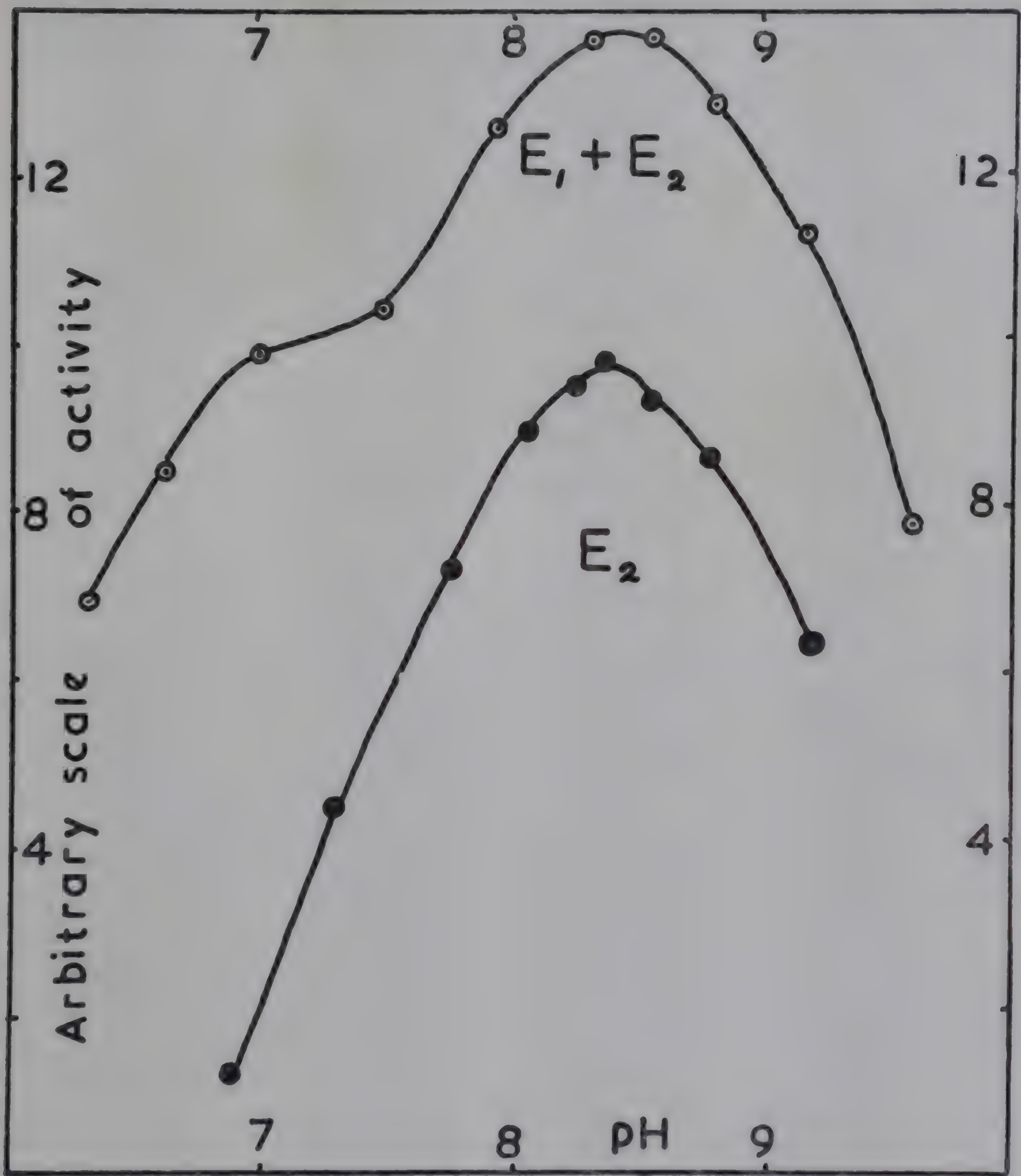

FIG. 4
Curves relating the activity of fraction E_2 and fractions E_1 plus E_2 to pH.

This could also be shown by pre-treatment of the substrate with E_1 at a range of pH values, followed by treatment with E_2. Under these conditions E_2 was found to be more active against those preparations which had had E_1 treatment in the regions of pH 7.8. The

addition to E_1 of small concentrations of E_2 permitted the solubilization to proceed. It was found that under these conditions the ratio of polysaccharide to protein liberated by E_1 containing E_2 was between 2 and 3 times as great as that liberated by E_2 either alone or in the presence of a small concentration of E_1 (Hall, 1956). This appeared to afford evidence for the involvement of E_1 in a reaction with a polysaccharide fraction of the substrate, although the fact that no polysaccharide appeared in solution without the intervention of E_2 would imply that E_1 does not sever sufficient bonds to liberate carbohydrate. The evidence, therefore, indicates that the two active fractions of elastase are respectively specific for a protein and a polysaccharide portion of the total fibre, but in both cases the enzyme is highly specific in its action, since no generalized proteolytic or mucolytic activity can be discerned.

The necessity for both protein and polysaccharide to be attacked before elastin can pass in solution affords evidence for the existence of a polysaccharide-protein complex in the fibre.

CHEMICAL EVIDENCE FOR A MUCO-PROTEIN COMPLEX

Further evidence in favour of this can be obtained by an examination of extracted fractions obtainable from whole *ligamentum nuchae* (Lloyd, 1956). After the removal of a large proportion of the mucopolysaccharide from minced ligament by extraction with hot 5 per cent sodium chloride solution a further fraction can be extracted by neutral 10 per cent calcium chloride solution. This material can be subsequently divided into two parts by dialysis. One of these is insoluble in water. This material must presumably undergo a certain amount of denaturation during dialysis since it no longer dissolves in calcium chloride solution nor will it dissolve in buffer solutions up to pH 9. However, on incubation with elastase it passes rapidly into solution and dissolution is accompanied by the release of very few free reducing groups indicating that the enzyme does not cause generalized breakdown of glycosidic linkages during reaction. Whereas whole *ligamentum nuchae* has a polysaccharide content of between 1 and 2 per cent and a preparation of elastin which has been decollagenated by treatment with acetic acid contains between 0.3 and 0.5 per cent polysaccharide, this fraction has a polysaccharide content of between 6 and 8 per cent. The amino-acid composition of the protein moiety of this preparation differs

from that of both collagen and elastin. It has a high proline content, relatively high acidic and basic amino-acid content and a hydroxyproline content intermediate between that of elastin and collagen. It could not, however, be a mixture of collagen and elastin since there are one or two spots in the region of methionine and tryptophan which could not be identified with components of either of the possible parent substances. Further analysis of the whole preparation showed that it contained about 38 per cent of reducing material on hydrolysis and about 2.4 per cent of hexosamine. Corresponding hydrolyses of a chondroitin sulphuric acid preparation give a ratio of reducing sugar to hexosamine of 13.4 to 15.2 indicating that the polysaccharide portion of the material extracted from elastic tissue was relatively devoid of hexosamines.

CONCLUSIONS

In 1952, owing to a belief that the elastase preparations then available were far purer than they have since proved to be, it was suggested that elastase attacked an outer matrix or sheath of the elastin fibre which protected an inner fibrous structure (Hall, Reed and Tunbridge, 1952). On the removal of this protective coating, the inner portion, which was assumed to be completely protein in nature, passed spontaneously into solution in the buffer.

More recent observations have done little to detract from the original concept of the elastin fibre as a dual structure and in this the chemical evidence has been amply borne out by electron microscope studies on elastin which has been subjected to the action of elastase (Hall, Reed and Tunbridge, 1955) on the one hand and by a study of the apparent production of elastin from collagen by action of alkali, collagenase and periodic acid on the other (Burton, Hall, Keech, Reed, Saxl, Tunbridge and Wood, 1955). The only variation from the original concept concerns the stability of the inner fibrous protein structure. This would appear to be the substrate for the enzyme E_2, whereas the polysaccharide portion of the amorphous outer coating is the substrate for E_1.

In the earlier paper by Hall, Reed and Tunbridge (1952) it was suggested, so as to conform with the observations of Adair, Davis and Partridge (1951) and Bowen (1953) that the outer amorphous mucoprotein portion might be considered as consisting of protein units of the order of 5000–10,000 molecular weight bound together

by the small amount of mucopolysaccharide present, while the inner fibrous component was totally composed of protein and consisted of a linear polymer of this protein fraction. This theory requires very little alteration in view of the mass of evidence which has accumulated since it was first proposed, but it is possible to see how other workers using different techniques for purification of elastin preparations have removed the whole of the polysaccharide and with it, therefore, at least part of the outer coating of the fibres, revealing the inner fibrous structures on which they have subsequently performed their chemical analyses. No doubt the analyses ascribed to this material can justly be claimed to be those of elastin; but only if elastin is defined as 'a protein derived from elastic tissue by certain given purification procedures'. Such a preparation would appear to bear little relationship to elastin as defined by histologists, which calls for the inclusion of a polysaccharide fraction to account for certain of its histological properties.

In the questions dealt with above concerning the involvement of polysaccharide in the stabilization of the elastin fibre, the tacit assumption has been made that the protein portion of the fibre consists of identical fragments but that the units concerned are combined with polysaccharide in the amorphous phase and only with one another in the fibrous phase. This is in complete agreement with the earlier observations of Partridge and co-workers (1951) but the observations of Lansing, Roberts, Ramasarma, Rosenthal and Alex (1951) cannot be correlated with this concept since they call for the existence of elastins having different amino-acid compositions in samples of elastic tissue from different age groups. A possible explanation of this has been afforded by the observations of Hall, Keech, Reed, Saxl, Tunbridge and Wood who suggested the possibility that their *in vitro* observations on the conversion of collagen to elastin could be extrapolated to cover the *in vivo* field (1955). Degeneration of collagen fibres prior to the synthesis of elastin fibres would explain why various elastin preparations appear to contain amino acids over and above those normally associated with the classical elastin analyses since the breakdown of collagen from the older age groups is not so complete and collagen fibres which have not degraded to the extent necessary for the production of classical elastin will become embodied in the forming elastic fibre. Some of the collagen fibres do apparently, however, convert completely to elastin, since Hall has shown (1951) that the extraction

with urea solution of aortic elastin preparations which have an amino-acid pattern between that of collagen and elastin produces a product in the penultimate stage before dissolution having an amino-acid composition in close conformity to that of classical elastin.

It appears, therefore, as if there may not be a single entity 'elastin' but rather a series of elastins differing in their amino-acid composition and dependent for their varying analyses on the age and hence reactivity of the collagen from which they are derived.

A model for the structure of elastin based on the incomplete evidence as yet at our disposal must of necessity be rather tentative, but on the basis of the foregoing observations it would appear feasible to propose a structure for elastin which can be diagrammatically represented as in Fig. 5. Here in agreement with the suggestions put forward earlier (Hall, Reed and Tunbridge, 1952) the fibre is considered as consisting of a two-phase system, both outer and inner phases being composed of identical protein units, the outer ones being linked through a small amount of polysaccharide, whereas the inner ones are joined directly in a linear array by bonds of a different nature. This concept will explain the observations of Partridge and Davis (1955) regarding the indentical amino-acid composition of the various fractions obtained from elastin by oxalic acid hydrolysis. The protein unit would be identifiable as the β-fraction, having a molecular weight of 5500 whereas the major α-fraction would represent linear polymers of this smaller sub-unit linked by bonds which were not easily broken by oxalic acid. Here again the model affords an explanation of two further observations of Partridge and his co-workers, namely that the quantity of the β-fraction present was relatively small. This is as would be expected if prolonged autoclaving removes such a high proportion of the polysaccharide. Secondly, Partridge has noted that the β-fraction is released preferentially in the earlier stages of attack, whereas the α-protein is liberated preferentially only in the later stages of the reaction. These observations are easily explicable if one accepts the hypothesis that the β-fraction represents a portion of the outer sheath, while the α-fraction is the substance of the inner fibrous core.

The author wishes to acknowledge the active help and sustained interest of Professor R. E. Tunbridge. He also wishes to thank Dr. G. C. Wood and Miss H. Saxl for much helpful discussion.

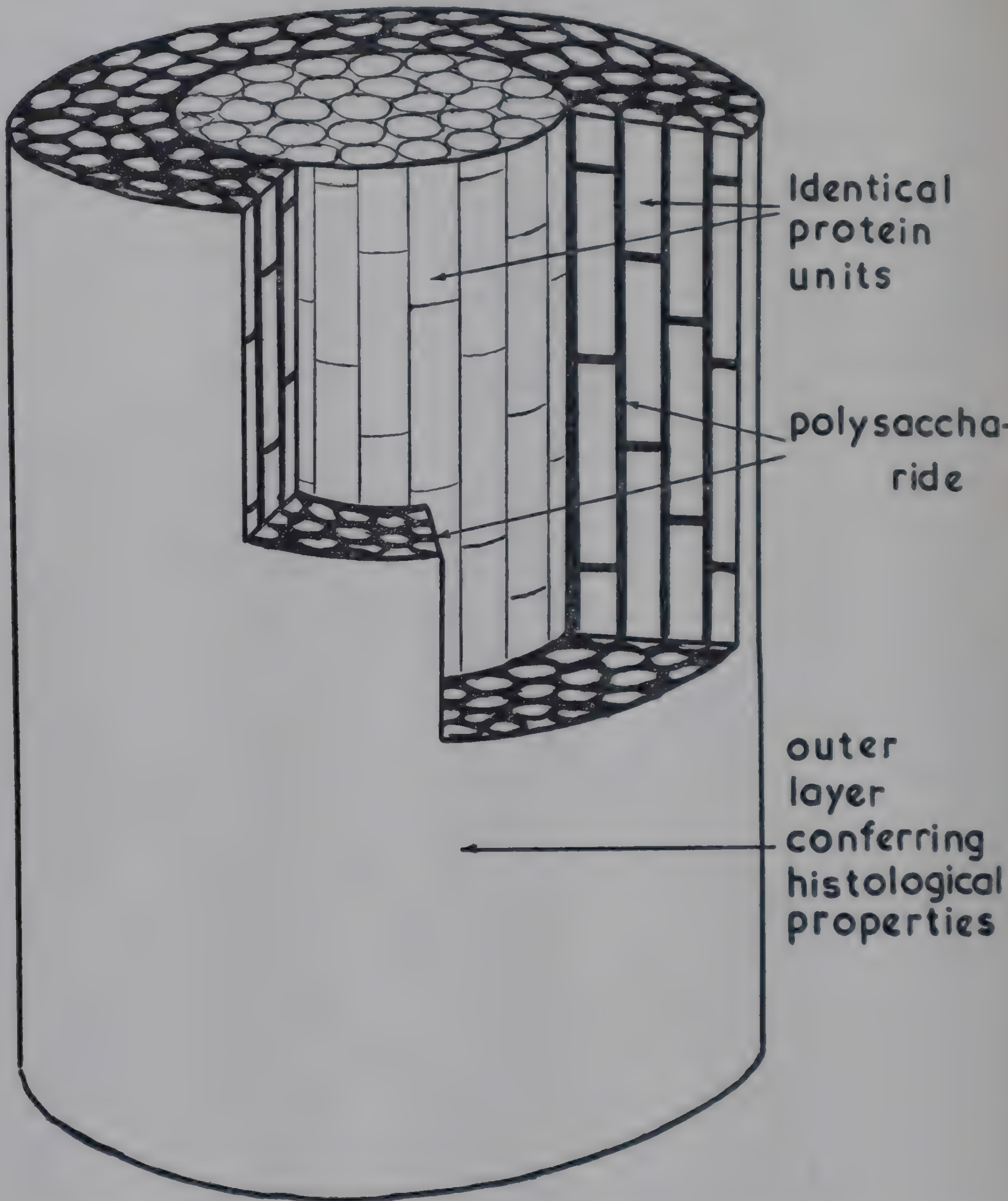

FIG. 5

An idealized representation of the author's suggested model for an elastin fibre. The protein units making up the whole of the inner core and the majority of the outer core are identical and are directly linked in the former phase, but are linked through polysaccharide in the latter phase.

GROUP DISCUSSION

In reply to Dr. Snellman, DR. HALL said that it was possible to absorb E_1 on to the surface of elastin but he did not know whether it could be precipitated by means of a polysaccharide.

Dr. Sylvén asked Dr. Partridge if his fractions were tested by the usual staining reactions for elastin, collagen and others, and Dr. Partridge replied that he had not investigated the staining reactions of the soluble materials. His purified fibres, however, were stained with orcein like elastic tissue, and were not stained by basic and acidic dyes.

Dr. Gillman, referring to Dr. Hall's model of the elastin fibre, asked if it was the polysaccharide in the outer coating that was responsible for the staining properties of the material. Dr. Hall replied that the staining reactions might equally well be due to protein groupings in the outer coating which were not available in the core.

What was needed, said Dr. Gillman, was an enzyme which would specifically strip off the outer coating to see if at the same time this removed staining properties. Dr. Hall replied that this was just what was observed when elastase was used.

Dr. Meyer said the purified polysaccharides which he had isolated from *ligamentum nuchae* are not acted upon by crude elastase prepared by Banga's procedure or by a mould enzyme mixture containing elastase but that he did not claim to have isolated all the polysaccharides present in *ligamentum nuchae*.

Referring to the insolubility of the polysaccharide described by Dr. Hall, he said that insolubility was characteristic of mucoids extracted from many tissues and was probably due to denaturation. He referred to the unreliability of reducing value measurements on hydrolysates of mucopolysaccharides as a measure of their reducing sugar content.

Dr. Hall replied that measurements of reducing power had been made only to show that the polysaccharides isolated from *ligamentum nuchae* did not contain CSA. Dr. Meyer did not think this conclusion was valid. In reply to Dr. Jackson, Dr. Hall said that trypsin has no effect on the mucopolysaccharide of *ligamentum nuchae*.

Dr. Partridge said it would be interesting to test Hall's enzyme system on his own elastin preparation. He did not think, and Dr. Hall agreed, that E_1 is necessarily a polysaccharide-splitting enzyme. He was prepared to accept the idea that the elastin fibres are coated by a polysaccharide protein complex but did not think that the nature of the protein component was yet settled.

There was some discussion on nomenclature in which it was suggested that the term 'elastin' be used to describe the fibrous protein whereas the terms 'elastic tissue' and 'elastica' should be used to describe the fibres as they appear in the tissue.

THE STRUCTURE AND CHEMICAL COMPOSITION OF CONNECTIVE TISSUE

I. Banga and J. Baló

Connective tissue fibres showing homogeneity under the light microscope and homogeneity by histological standards represent a chemically complex system containing many components. Moreover the functioning native fibre can be taken as a biological entity in which the chemically different components are held together by true chemical bonds or by van der Waals forces. If some of the bonds are loosened or broken, the fibre will show altered function and the physico-chemical properties of the fibre will also be different from the normal. No fibre is capable of functioning unless its chemical structure is intact.

The present work deals with the mucoprotein components of connective tissue fibres and with those enzymes which take part in the degradation of the fibre. The mucoproteins and mucopolysaccharides bound to the fibres – although forming only a small part of these – greatly influence the physico-chemical behaviour, swelling, permeability and functional capability of the fibres. Our experiments show that some colloidal properties of the fibres, for example the acid and neutral swelling, seem to be related not only to the electrovalent or hydrogen bonds of the polypeptide chains but also to the cross linkages present between the mucoprotein and polypeptide chains. We believe that although the mucopolysaccharides form only a small part of the connective tissue fibre nevertheless their biological importance is very great.

ENZYMES CAPABLE OF DEGRADING CONNECTIVE TISSUE: ELASTASE, MUCOPROTEINASES AND HYALURONIDASE

The investigations of the complex structure of connective tissue fibres have become only recently possible now that some agents are at our disposal which act specifically upon some ingredients of the fibres. Through these agents the fibres can be decomposed gradually and the split products can be analysed separately.

Among the specific enzymes we would like to mention elastase (Baló and Banga, 1949, 1950) which was a very valuable agent in the investigation of the structure of connective tissue fibres on account of its properties of dissolving elastic and metacollagen fibres (Banga, Baló and Szabó, 1956). Partridge and Davis (1955) have shown that one of the components of the elastase enzyme complex is a proteolytic enzyme. We want to maintain the name elastase for this proteolytic enzyme mainly because we succeeded recently in isolating and crystallizing an enzyme which dissolves elastin and which was proved to be homogeneous by electrophoresis.

Hall, Reed and Tunbridge (1952) first described mucolytic properties of elastase. Hall (1953) produced evidence that elastase contains two enzymes one of which must be mucolytic. We corroborated this suggestion (Banga and Baló, 1956) by isolating from the pancreas two mucoproteinases which split specifically the mucoproteins bound to connective tissue but which do not digest the polypeptide chain. These mucoproteinases usually occur as contaminants in the elastase preparations.

The mucolytic enzymes of the pancreas are not homogeneous. So far we have shown by preparative methods as well as by electrophoretic examinations and by specific activity measurements on different substrates that there are at least three different mucoproteinases. One of these shows its greatest specific activity on the elastomucin component of elastic fibre; therefore we have termed it elastomucoproteinase. The specific substrate for elastomucoproteinase seems to be identical with the alkali-labile component of elastic fibre.

The second enzyme splits one mucoprotein component of collagen fibre, therefore it is called collagen-mucoproteinase. The third enzyme shows a specificity to the mucoprotein component of the blood serum: we call this enzyme serum-mucoproteinase (Banga, 1955). This group of mucolytic enzymes differs from the hyaluronidases and from the other known enzymes of the pancreas and represents a new group of enzymes. The isolation of mucoproteinase is in progress. So far we have only obtained one mucolytic enzyme in pure crystalline form and as an electrophoretically homogeneous protein. This is the collagen-mucoproteinase.

Besides elastase and mucoproteinases we have been able to demonstrate the activity of Hyason (Organon) on the collagen fibre. In this case a preliminary treatment with mucoproteinase is necessary.

THE DIFFICULTY OF MEASURING THE ACTIVITY OF MUCOPROTEINASES

In connection with the examination of mucoproteinases there are two kinds of difficulties. The first one is the question of mucoproteins as substrates. In connective tissue the polypeptide chains make up 95 per cent of the fibre and the amounts of mucoproteins are only 3-5 per cent. These mucoproteins cannot be liberated by means of chemical methods without causing an alteration in their molecules. Therefore one is obliged to measure the mucoproteinase activity on entire fibres, that is on a substrate in which 95 per cent of the substance is useless and the specific substrate is present only in a small proportion. In consequence, some of the classical enzyme methods, for example the determination of the substrate concentration curve, cannot be carried out.

Furthermore, even the small amount of mucoprotein associated with any single connective tissue fibre cannot be considered homogeneous. The elastic fibre as well as the collagen fibre contains two or three different mucoprotein or mucopolysaccharide components.

The second difficulty consists in the fact that there are no methods whereby the amount of a single mucoprotein associated with the fibre can be measured quantitatively. The sensitivity of the known methods for determination of carbohydrates is relatively small and is influenced by the presence of proteins to such an extent that their use for the quantitative measurement of the mucoproteins of connective tissue is impracticable. In order to overcome this difficulty we have applied for our purpose the quantitative Molisch reaction of Szára (1956) (Banga and Baló, 1956). 1-Naphthol-2-sulphonic acid is water-soluble and gives different colour reactions with the various mucoproteins. We measure, therefore, the light absorption of the coloured solution at several wave-lengths from 420 to 750 mμ with the Stuphophotometer (Zeiss) by the use of colour filters. The colour absorption curve thus obtained is specific for the single mucoprotein. On the basis of these experiments it seems that the mucoproteins of elastic and collagen fibres differ from one another. In consequence probably the enzymes are also different.

PROPERTIES OF MUCOPROTEINASE ENZYMES OF THE CONNECTIVE TISSUE FIBRES

According to the difficulties mentioned above we know very little about the properties of the mucoproteinases of connective tissue. Table I summarizes the present data relating to the mucoproteinases isolated from bovine pancreas. We call these enzymes mucoproteinases because they split off the fibres not only carbohydrates but also mucoproteins as judged by the appearance in solution of large amounts of undialysable nitrogen. This nitrogen cannot be derived from hexosamine because the latter is present only in small quantity. The mucoproteinases split besides the mucoprotein components also a dialysable carbohydrate component containing nitrogen. The latter component is approximately 20-25 per cent of the components split by the enzymes. Due to the small quantities split, no further analytical results are available up to now.

TABLE I

	MUCOPROTEINASES		
Enzymes	*Substrate*	*Stability*	*End-products*
Elasto-Mucoproteinase	Cervical Ligament (Collagen-free) Aorta (Collagen-free)	2 components: Thermolabile + Thermostable	2 components Undialysable Mucoprotein (75 per cent) + Dialysable sugar (25 per cent)
Collagen-Mucoproteinase	Achilles Tendon Rat Tail Tendon Procollagen Metacollagen	2 components: Thermolabile + Thermostable	2 components Undialysable Mucoprotein (75 per cent) + Dialysable Sugar (25 per cent)
Serum Mucoproteinase	Serum protein	Thermolabile	Mucoprotein Sugar?

The data of Table I show that besides the thermolabile components both the elastomucoproteinase as well as collagen-mucoproteinase contain also heat-stable components. So far it is undecided whether these components found in both of the enzymes are identical or not.

THE FRACTIONAL DECOMPOSITION OF MUCOPROTEINS, I.E. MUCOPOLYSACCHARIDES OF COLLAGEN FIBRES

Table II summarizes our results according to which the collagen fibre – as it has a complex constitution – yields different structures by applying different chemical substances or enzymes as degradation agents.

According to our still incomplete knowledge the native collagen fibres contain, besides procollagen and metacollagen, also chondromucines comprising the chondroitin sulphate B and C (CHS. B, C). Furthermore, it contains two other mucoproteins termed mucoprotein$_1$ and mucoprotein$_2$ (Banga and Baló, 1956).

Among the different effects on the collagen fibres (cf. Table II, 1) the effect of heat and that of 40 per cent KI solution will be discussed, first as they cause the native collagen fibres to contract and relax (Banga, Baló and Szabó, 1954). The relaxed collagen consists of metacollagen still containing a reducing mucopolysaccharide, mucoprotein$_2$. The metacollagen is elastic and can be dissolved entirely by elastase.

As is shown in Table II, 2, the effect of mucoproteinases is to produce a kind of collagen fibre – called collagen residuum – which is not dissolved by elastase. It cannot do work connected with the contraction-relaxation phenomenon. It tears after contraction.

In Table II, 3, we describe the effect of hyason in the presence of physiological solution of NaCl and call the fibre again collagen residuum. In our opinion this fibre differs from the native fibre only by not containing CHS. C. This lack does not influence the stability of the fibre because it is capable of showing the phenomenon of contraction and relaxation. We should like to note that in the experiment described by Jackson (1954) the hyaluronidase was dissolved in acetate buffer of pH 5.2 containing 0.15 M NaCl which solution will dissolve the procollagen even without hyaluronidase and will cause a decrease in the stability of the fibre. Therefore in the experiments of Jackson two kinds of effect come into action: the effect of buffer which dissolves the procollagen and the effect of hyaluronidase which dissolves the CHS. C from the collagen fibre.

The experiments in Table II, 4 and 5 reveal interesting data. In the ground substance of tendon, CHS. B and C and hyaluronate are present (Meyer, 1954) and are dissolved by the action of hyaluronidase. Our experiments have shown that by avoiding the use of acid

TABLE II

FRACTIONAL DEGRADATION OF COLLAGEN

Effects	*Substrate*	*Constituents*	*End-product*	*Constituents*	*Elastase solubility*	*Function: capable of contraction relaxation*
(1) Heat effect or 40 per cent KI	Native Collagen Fibre of Rat Tail tendon	Procollagen Metacollagen Chondromucin (CHS. B + C) $Mucoprotein_1$ $Mucoprotein_2$	Metacollagen (Elastic)	Metacollagen $Mucoprotein_2$	Soluble	No contraction (Synerisis)
(2) Mucoproteinase	,,	,,	Collagen Residuum	Procollagen Metacollagen CHS. B + C	None	After contraction tears
(3) Hyason (Organon)	,,	,,	Collagen Residuum	Procollagen Metacollagen $Mucoprotein_1$ $Mucoprotein_2$ CHS. B	None	Contraction—Relaxation remains unchanged
(4) Mucoproteinase (first) Hyason (after)	,,	,,	Collagen Residuum (elastic)	Procollagen Metacollagen CHS. B	Degraded to filaments	No contraction
(5) Hyason (first) Mucoproteinase (after)	,,	,,	Collagen Residuum	Procollagen Metacollagen CHS. B + C?	None	After contraction tears
(6) pH4 Citrate Extraction and 1h 37° C. H_2O	,,	,,	Collastromin	Metacollagen $Mucoprotein_2$ CHS. B + C	None	No contraction

buffer which dissolves the procollagen, as mentioned above, there is very little polysaccharide which can be dissolved by Hyason. In consequence, there is no change in function of the fibre. But if the collagen fibre is previously treated with pancreatic mucoproteinase and afterwards by Hyason (T.R. 2.5/ml.) then it can be shown that Hyason will also dissolve a mucoid that changes the stability of the fibre. After the effect of both enzymes, a fibre remains which has elasticity confirming the views of Hall, Keech, Reed, Saxl, Tunbridge and Wood (1955) and of Burton, Hall, Keech, Reed, Saxl, Tunbridge and Wood (1955) on the transformation of collagen into elastin. Here the transformation is established through the effect of enzymes in contrast to the transformation caused by chemical substances. This elastic collagen is not dissolved without residue by elastase but it disintegrates into filaments. If the order is changed and the fibre is treated first with Hyason and afterwards with mucoproteinase then the effect is different and is similar to that found when the fibres were treated with mucoproteinase alone. This experiment shows that Hyason can have a profound effect on the collagen fibre only if another mucoid has been dissolved previously. The dissolution of the latter is completed by the enzyme collagen mucoproteinase. If native collagen fibres are completely extracted at pH 4 with citrate to remove procollagen (Orekhovitch *et al.*, 1948) and treated further with water for one hour at 37° C, the collastromin of Tustanowski *et al.* (1954), is obtained. The collastromin contains, besides a polypeptide chain, mucoproteins and CHS. A, B and C. Collastromin is insoluble in elastase and does not show heat or chemical contraction.

THE EFFECT OF AGE ON THE COMPOSITION OF MUCOPROTEINS OF COLLAGEN FIBRE

It has been shown that the mucoprotein composition of collagen fibres varies according to age. For the demonstration of the differences the Szára reaction mentioned above is especially suitable. The main qualitative difference between young and old fibres is revealed by the fact that from the collagen fibres of young animals mucoproteinase releases a substance which gives with the Szára reagent a red colour with green fluorescence. The fibres of old animals fail to show this fluorescence. The fluorescent component disappears step by step from the fibre with age and cannot be demon-

strated in very old animals. The other difference manifests itself if the treatment shown in Table II, 4 and 5 is applied to the collagen fibres derived from animals of different ages. The effect of Hyason after the treatment by mucoproteinase releases components giving different colours with the Szára reagent. After mucoproteinase treatment, Hyason dissolves from young fibre, 2–3 weeks old, a component which gives with the Szára reagent a colour with a maximum absorption at 530 mμ. In old animals the same component has its maximum absorption at 570 mμ. These differences show the change with age in the quality of mucoproteins in the fibres.

DISCUSSION

During the last few years, the number of papers dealing with the association between polysaccharides, chondroitin sulphates and hyaluronates and the proteins of collagen have increased. We would particularly like to refer to the work of Highberger, Gross and Schmitt (1951, 1953, 1954) who in their electron microscopic studies produced evidence on the genesis of fibrils from acid soluble collagen. Delaunay, Bazin, Fauve and Henon (1956) proved experimentally the association between the mucopolysaccharides of umbilical cord and collagen and have suggested the existence of strong linkages. These authors believe that these linkages play also an important role *in vivo*. Grassmann and Kühn (1955) have also shown that procollagen contains mucopolysaccharides. We could also demonstrate with the Szára reaction (unpublished data) that procollagen contains a large amount of mucopolysaccharides (6–8 per cent) which remains in the molecule after purification.

In the present work we have given further evidence on the existence of specific enzymes acting on the mucoproteins of connective tissues and we think that the mucoproteins present in connective tissue fibres play an important role. Histologists have for a long time paid special attention to the metachromasia of connective tissues as well as to Schiff's periodate reaction; changes in these are connected with pathological conditions. The discovery of hyaluronidase, i.e. of the spreading factor, gave a great impetus to the research on mucopolysaccharides of connective tissue. However, most studies have so far been restricted to the ground substance. Our study attaches importance to the analysis of the fibre and endeavours

to establish conditions for examining the mucoproteins bound to the polypeptide chain of the fibres. Through the possession of pancreatic mucoproteinases we hope to gain a clearer picture of the relation between the constituents of the connective tissue fibre.

SUMMARY

(1) Bovine pancreas contains, in addition to elastase which has proteolytic activity on connective tissue fibres, mucolytic enzymes as well which act on the mucoproteins. These enzymes can be looked upon as a new group of enzymes which differ from the known pancreatic enzymes and seem to be specific for the mucoproteins of connective tissue and of serum protein.

So far we have distinguished three different enzymes in this group and called them after their specific substrates: elastomucoproteinase, collagen mucoproteinase and serum mucoproteinase respectively.

(2) The difficulties of examining mucoproteins and mucopolysaccharides have been discussed and a method was described for their examination.

(3) The mucoproteins bound to the fibres were shown to vary qualitatively according to the age of the animals.

(4) The gradual enzymic release of the different mucoproteins from collagen fibres leads to an alteration of their function.

GROUP DISCUSSION

In reply to Dr. Orekhovitch, Dr. Banga said that procollagen and metacollagen are not identical but that metacollagen resembles the collastromin of Tustanowski. However, unlike metacollagen, collastromin is not dissolved by elastase.

Answering Dr. Meyer, Dr. Banga said that in addition to the Molisch and the orcein methods she had used the anthrone method to determine dialysable sugars but she did not know whether or not they were monosaccharides. She pointed out that the dialysate itself also contains nitrogen.

In reply to Dr. Hall, Dr. Banga said that all the mucoproteinases that she had used came from the pancreas.

In reply to Dr. Meyer, Dr. Banga stated that in studying the serum mucoproteinase, the substrate native serum was not dialysed and the substrate was not isolated from the serum, in order to avoid denaturation. The ethanol precipitated serum which could be looked upon as a denatured substrate was also attacked by the enzyme and, indeed, at a greater

rate than the native serum, and this she attributed to the presence of inhibitors in the serum which were removed by ethanol. In reply to Dr. Snellman, DR. BANGA said that that she had not yet isolated the acid mucoprotein from serum.

In reply to Dr. Grassmann, DR. BANGA said that the collagen she had used was alcohol-dried Achilles tendon and also Achilles tendon which had been treated with sodium hydroxide. The resulting material contained 0.6 per cent to 0.9 per cent polysaccharide.

DR. GRASSMANN said that he had always obtained similar figures for the carbohydrate content. He then asked how many per cent of protein nitrogen were dissolved from the collagen and what was the relation between the sugars and the nitrogen in the dialysable part.

DR. BANGA answered that she determined only the polysaccharide of the dialysable and the undialysable part.

There was some discussion between Dr. Grassmann and Dr. Meyer regarding the reliability of methods for estimating sugars in hydrolysates of mixtures of polysaccharide and protein. DR. GRASSMANN said that a more reliable estimation could be made by using mild hydrolysis conditions and separating the amino acids from the sugars as their DNP derivatives by column chromatography. Dr. Meyer was sceptical.

THE COMPOSITION OF SOME PROTEIN FRACTIONS ISOLATED FROM BOVINE SKIN

J. H. Bowes, R. G. Elliott and J. A. Moss

Investigations by various groups of workers have shown that the collagen of the skin and other tissues can be subdivided into a number of different fractions of varying solubility, while evidence for the presence of one or more non-collagenous type proteins closely associated with the collagen is accumulating. Eastoe and Eastoe (1954) have demonstrated the presence of a protein, similar in composition to the serum proteins, in the mucopolysaccharide fraction isolated from bone collagen. The work of Consden and his collaborators (Consden, 1953; Consden, Glynn and Stanier, 1953) indicates the presence of a similar constituent in connective tissue and rabbit skin. It seems probable that this protein-polysaccharide fraction represents the main constituent of the ground substance.

During recent years the work in these laboratories has been concerned with the separation of various constituents from animal skin and determination of their chemical composition. Interest has primarily been centred on those constituents which are extracted from skin by dilute acid and alkaline solutions, and this paper summarizes the results of these investigations, and discusses their significance in relation to the structure of collagenous tissue.

The work has been mainly carried out on the skins of the larger animals which are used for leathermaking. It is obvious from our results that the relative amount of collagen and non-collagenous protein constituents varies from one animal to another and with the age of the animal, and also that the amounts and identities of the sugars present may vary with the origin of the collagenous tissue. How far the present results are applicable to human skin is, therefore, a matter of conjecture.

ACID-SOLUBLE COLLAGEN

Acid-soluble collagen was prepared from the skin of a 6- to 8-week-old bull calf as described by Orekhovich, Tustanowski, Orekhovich and Plotnikova (1948). The precipitate was purified

by redissolving in citrate buffer and dialysing against tap water. The amino-acid composition of the purified protein determined by the method of Moore and Stein (1951) is given in Table I, together

TABLE I

AMINO-ACID CONTENT OF OX-HIDE COLLAGEN AND OF CITRATE-SOLUBLE COLLAGEN OF CALF SKIN (FROM BOWES, ELLIOTT AND MOSS, 1955)

	Citrate-soluble protein			*Ox-hide collagen*		
	N as per cent protein-N	*gm./100 gm.*	*gm. residues/100 gm.*	*N. as per cent protein-N*	*gm./100 gm.*	*gm. residues/100 gm.*
Total N	—	17.70	—	—	18.60	—
Amino N	2.65	0.49	—	2.50	0.46	—
Glycine	27.48	26.07	19.81	26.66	26.57	20.20
Alanine	8.84	9.95	7.94	8.72	10.32	8.23
Leucine	1.93	3.20	2.76	2.14	3.73	3.22
Isoleucine	0.84	1.39	1.20	1.08	1.88	1.62
Valine	1.53	2.26	1.91	1.58	2.46	2.08
Phenylalanine	0.95	1.98	1.78	1.07	2.35	1.95
Tyrosine	0.22	0.50	0.45	0.41	0.99	0.89
Tryptophan	—	—	—	—	—	—
Serine	3.19	4.23	3.51	3.06	4.27	3.54
Threonine	1.47	2.21	1.87	1.43	2.26	1.92
Cystine	—	—	—	—	—	—
Methionine	0.41	0.78	0.68	0.49	0.97	0.85
Proline	8.95	13.02	10.98	9.43	14.42	12.16
Hydroxyproline	8.22	13.62	11.75	7.37	12.83	11.07
Arginine	15.16	8.34	7.48	14.22	8.22	7.37
Histidine	0.45	0.29	0.26	1.02	0.70	0.62
Hydroxylysine	0.88	0.90	0.80	0.93	1.00	0.89
allo Hydroxylysine*	0.38	0.39	0.34	0.14	0.15	0.13
Lysine	3.87	3.57	3.13	4.08	3.96	3.47
Aspartic acid	3.60	6.05	5.23	3.93	6.95	6.01
Glutamic acid	5.93	11.02	9.69	5.69	11.16	9.75
Amide	2.92	0.52	—	3.50	0.66	—
Hexosamine	—	0.01	—	—	0.05	—
Total	97.30	—	91.56	96.97	—	95.97
Average residue weight:						
By summation	—	—	90.5	—	90.9	—
By N-distribution	—	—	95.5	—	91.2	—

* Not identified, but assumed to be *allo*hydroxylysine for purposes of calculation.

with that of collagen prepared from the middle layer of a fresh ox hide as described by Bowes and Kenten (1948). (For full details of the preparation of acid-soluble collagen and of the amino-acid determinations, see Bowes, Elliott and Moss, 1955.)

The nitrogen content of the citrate-soluble collagen was appreciably lower than that of the ox-hide collagen and it was, therefore, considered preferable to compare the composition of the two proteins on the basis of amino-acid nitrogen as a percentage of the total nitrogen, rather than as weight of amino acid per hundred parts of protein.

The composition of the two proteins is essentially the same, but there are a number of small differences. The amide, tyrosine and histidine contents of the citrate-soluble collagen are definitely lower, and the leucine, isoleucine, and possibly aspartic acid, contents are slightly lower than in the ox-hide collagen, while the hydroxyproline content is higher. The hexosamine and hexose content is even lower than that of the ox-hide collagen.

TABLE II

TERMINAL RESIDUES IN CITRATE-SOLUBLE COLLAGEN (FROM BOWES, ELLIOTT AND MOSS, 1955)

	Citrate-soluble collagen	*Gelatin from citrate-soluble collagen*
	(m-moles/100gm. DNP—protein)	
Aspartic acid	0.06	0.14
Alanine	0.04	0.20
Glycine	—	0.14
Glutamic acid	—	0.17
Serine	—	Trace
Threonine	—	Trace

About 97 per cent of the total nitrogen is accounted for with both proteins, but, whereas with the ox-hide collagen the sum of the amino-acid residues adds up to 96 and the deficit could be due to overall losses of amino acids during analysis, with the citrate-soluble collagen the sum of the amino-acid residues is only 91.6. Even if all the nitrogen unaccounted for was present in amino acids of high molecular weight, the total of the residue weights would still fall appreciably short of a hundred. This, together with its low nitrogen content compared with ox-hide collagen, suggests the presence of about 4 to 5 per cent of some non-protein constituent of low nitrogen content. Attempts to detect such a constituent have so far failed. Determination of reducing sugars and hexosamine, as well as paper chromatography, indicate the presence of only very small amounts

of hexosamine, galactose, glucose and mannose, amounting in all to less than 0 1 per cent, so that no appreciable amount of polysaccharide or carbohydrate is present (Moss, 1955). The possibility that fat might be present as in reticular tissue (Windrum, Kent and Eastoe, 1955) was also investigated, but procedures similar to those employed by Windrum *et al.* (1955) indicated the presence of less than 0.1 per cent of fat.

The N-terminal residues of the ox-hide collagen, the citrate-soluble collagen and the gelatin obtained from it were determined using 1- fluoro-2: 4-dinitrobenzene (Sanger, 1945). No N-terminal residues were detected in the ox-hide collagen using this technique, and in the citrate-soluble collagen the amounts were very small (Bowes and Moss, 1953). It is, perhaps, of interest that the N-terminal residues found in ox-hide collagen after treatment with hyaluronidase were the same as those found in the citrate-soluble collagen (Bowes and Moss, 1953). Unfortunately it is not certain that the hyaluronidase preparation used was completely free from proteolytic activity so that release of these end groups cannot with certainty be ascribed to the removal of polysaccharide. On warming to 40° C for a few minutes, further end groups were released from the citrate-soluble collagen and on cooling the solution set to a gel.

It is possible that the differences in the composition of ox-hide collagen and the citrate-soluble collagen found in this investigation are due to variations in age and breed. In view, however, of the evidence available regarding the effect of various treatments on the composition of collagenous tissue (Bowes, Elliott and Moss, 1953; Consden, 1953), and for the presence of a non-collagenous protein in the mucopolysaccharide fraction obtained from bone collagen and connective tissue (Eastoe and Eastoe, 1954; Consden, Glynn and Stanier, 1953), it seems more probable that these small differences in composition are due to the presence, in close association with the adult collagen, of a protein constituent which is relatively rich in amide-nitrogen, tyrosine, histidine and to a less extent leucine, isoleucine and possibly aspartic acid, and low in hydroxyproline compared with the citrate-soluble collagen.

Some preliminary experiments on the extraction of tendon with citrate buffer suggested that a protein fraction of low hydroxyproline and high tyrosine content was extracted together with the collagen and was left in solution after precipitation of the collagen with sodium chloride. It was, therefore, decided to investigate

further this, and other fractions extracted from calf skin by dilute acid and alkaline solutions.

EXTRACTION OF CALF SKIN WITH DILUTE ACID AND ALKALINE SOLUTIONS

Extraction Procedure

A fresh skin from a bull calf about 5 weeks old was used for this investigation. In order to reduce complications due to contamination with keratinous material, elastin, muscle fibres, sebaceous glands and other cellular material, only the middle layer of the skin was used. This was obtained by sectioning 1-inch punches on a freezing microtome. The first few sections from the flesh side were discarded and the following sections collected until the base of the hair follicles was reached. The sections were transferred immediately to 0.1 M disodium hydrogen phosphate solution, pH 9.1 and stored below 4° C. In all about 470 punches were sectioned and the wet weight of the sectioned skin obtained was about 400 gm. The sections of skin were extracted five times with approximately five times their weight of 0.1 M phosphate buffer, pH 9.1, to remove, as far as possible, the plasma proteins. The final phosphate extract contained less than 0.02 mg. nitrogen per ml. corresponding to less than 0.1 per cent of the total-nitrogen of the skin. The macerate was then extracted seven times with four times its weight of 0.1 M citrate buffer, pH 3.7, until the nitrogen content of the extracts again fell to a low value (0.07 mg. N/ml.). The citrate buffer was followed by ten extractions with 0.05 M acetic acid adjusted to pH 2.8 with hydrochloric acid. The macerate was finally neutralized, and extracted seven times with sodium hydroxide solution, pH 12.0 to 12.3. All extractions were carried out at 0° to 4° C, and were, in general, each of 24 hours' duration. The acid-soluble collagen was precipitated from the extracts by the addition of sodium chloride to a final concentration of 5 per cent. The filtrates from the citrate-buffer extracts, with the exception of the first, were combined, dialysed to remove salts and concentrated. The concentration was carried out in the same cellulose tubing used for dialysis by blowing a stream of air over these at room temperature. The acetic acid solutions were treated similarly. The alkaline extracts were neutralized, concentrated and precipitated with three volumes of ethanol.

About 8.5 per cent of the total-nitrogen of the skin was extracted

with phosphate buffer, 20 per cent with citrate buffer, another 8.5 per cent with dilute acetic acid, and 2.2 per cent with dilute alkali, leaving a residue of about 60 per cent. In the citrate buffer and acetic acid extracts nearly all the protein was precipitated by sodium chloride leaving only very small amounts of nitrogen in solution, representing about 0.3 and 0.2 per cent of the total-nitrogen respectively. Of this material remaining in solution only about one-quarter was recovered after dialysis and concentration. In the alkaline extracts about half the nitrogen was precipitated by alcohol.

Amino Acid Composition of Protein Fractions

The hydroxyproline and tyrosine contents of some of the fractions were determined in order to gain information as to their identity (see Table III). The method of Neuman and Logan (1950) was used

TABLE III

HYDROXYPROLINE AND TYROSINE CONTENT OF SKIN FRACTIONS

	Hydroxyproline-N	*Tyrosine-N*	*Collagen-N**
	Amino or Imino-acid-N as per cent Total-N		
Citrate-Soluble Proteins			
Precipitated by NaCl			
Extract 1	7.68	0.32	93.4
2	7.95	0.19	97.0
4	7.88	0.29	95.8
7	8.15	0.26	99.3
Not precipitated by NaCl	0.1	0.56	1.2
Acetic Acid-Soluble Proteins			
Precipitated by NaCl			
Extract 2	7.47	0.30	90.6
4	7.73	0.28	94.2
6	7.86	0.27	93.8
7	7.66	0.30	93.3
10	7.48	0.31	90.4
Not precipitated by NaCl	0.19	1.67	2.3
Alkali-Soluble Protein			
Precipitated by ethanol			
Extract 2	0.42	2.33	5.1
4	0.93	0.91	11.3
5	1.51	1.08	17.7
6	1.58	1.54	19.3
Residue after extraction	7.62	0.31	92.9

* Calculated from the hydroxyproline content assuming a value of 8.22 for hydroxyproline—N expressed as a per cent of total collagen-N.

for the determination of hydroxyproline, and that of Udenfriend and Cooper (1952) for tyrosine. The proteins precipitated by the addition of sodium chloride to the acid extracts all had a high hydroxyproline and a low tyrosine content, and obviously consisted mainly of collagen. The protein recovered from the filtrates, however, had a very low hydroxyproline content and a relatively high tyrosine content. The protein fractions recovered from the successive alkaline extractions all had low hydroxyproline and high tyrosine contents suggesting that they mainly consisted of some non-collagenous protein.

An approximate value for the collagen content of the various fractions can be calculated from the hydroxyproline figures. In making this calculation a value of 8.22 for hydroxyproline-nitrogen as per cent total-nitrogen has been used. This is the highest value obtained in these investigations for a purified soluble collagen fraction, and it does not seem unreasonable to assume that it approaches that of 'pure' collagen, and that values lower than this indicate the probable presence of a non-collagenous protein. On this basis the precipitated proteins from the citrate buffer contain 96 to 99 per cent collagen, while those from the acetic acids contain rather less, 90 to 95 per cent. Presumably quite a high proportion of the non-collagenous protein present in these extracts has been carried down with the precipitate. On solution and reprecipitation the hydroxyproline content of the fractions was increased. In the alkali-soluble fractions the collagen content increases as the extraction proceeds suggesting that a non-collagenous type protein is preferentially extracted in the early stages.

The hydroxyproline content of the extracted skin residue is still lower than that of the purified citrate-soluble collagen and the tyrosine content is higher, suggesting that about 7 per cent of non-collagenous protein may still be present in this material.

The amino-acid composition of the three non-collagenous protein fractions was determined by the method of Moore and Stein (see Table IV). All values are given as amino-acid nitrogen expressed as a percentage of the total-nitrogen. The composition of the citrate-soluble and acetic acid-soluble fractions obviously differ considerably from that typical of collagen. In addition to the higher tyrosine and lower hydroxyproline content already noted, the glycine, proline and arginine contents are much lower, and the lysine, histidine, aspartic acid, leucine, isoleucine and valine contents are much

TABLE IV

THE AMINO-ACID COMPOSITION OF PROTEIN FRACTIONS EXTRACTED FROM CALF SKIN

	Citrate-Soluble Protein		*Acetic Acid Soluble Protein not precipitated*	*Alkali-Soluble protein*
	*Precipitated**	*Not precipitated*		
		(Amino Acid–N as per cent Total-N)		
Glycine	27.48	6.18	4.44	6.39
Alanine	8.84	8.46	5.70	5.13
Leucine	1.93	5.03	4.49	7.31
Isoleucine	0.84	2.98	1.96	3.81
Valine	1.53	4.30	3.21	4.76
Phenylalanine	0.95	2.18	1.88	2.65
Tyrosine	0.22	0.58	1.67	2.33
Tryptophan	not found	not determined		
Serine	3.19	4.01	3.76	4.77
Threonine	1.47	3.99	3.63	3.73
Cystine		not found		
Methionine	0.41	0.23	0.23	0.57
Proline	8.95	2.86	not found	4.17
Hydroxyproline†	8.22	0.10	0.19	0.42
Arginine	15.16	7.67	5.20	14.16
Histidine	0.45	2.56	5.64	3.62
Hydroxylysine	1.26	?	?	?
Lysine	3.87	7.07	5.03	7.75
Aspartic acid	3.60	7.28	7.14	8.48
Glutamic Acid	5.93	6.77	0.37	8.01
Ammonia (M and S)	(2.74)	12.78	9.56	(7.66)
Amide†	2.92	—	—	8.20
Glucosamine (M and S)	—	(0.18)	(7.27)	(0.63)
Galactosamine (M and S)	—	—	—	(1.02)
Hexosamine (Elson and Morgan)	0.01	1.33	10.14	2.90
Unknown‡	—	0.04	0.23	0.53
Totals (values in brackets not included)	97.23	86.40	74.47	99.69

* Taken from Bowes, Elliott and Moss, *Biochem. J.*, 1955, **61**, 143.
† Direct determinations.
‡ Calculated on the basis of 1 nitrogen atom per mole.

higher than in collagen. The two fractions differ from one another, particularly in their histidine, proline, glutamic acid, tyrosine, lysine, amide and hexosamine contents. The high amide content of these two fractions as indicated by the Moore and Stein procedure is difficult to reconcile with their dicarboxylic acid contents. It seems probable that appreciable amounts of ammonia must be derived from some source other than amide groups and degradation of serine and threonine. With the acetic acid-soluble fraction it is likely that some ammonia will have arisen from destruction of

hexosmine. The absence of any detectable proline and the very low value for glutamic acid in this fraction is also surprising. Low values for these acids were also found in the corresponding fraction of tendon examined in the preliminary experiment.

The high value for glucosamine in the acetic acid-soluble fraction was confirmed by independent determination of hexosamine by the Elson and Morgan method on a sample of the same hydrolysate. After mild hydrolysis with 2 N hydrochloric acid a rather higher value was obtained (Table V).

TABLE V

HEXOSE AND HEXOSAMINE IN CALF SKIN FRACTIONS

	gm. per 100 gm. Nitrogen			*gm. per 100 gm. Protein*	
	Hexose	*Glucosamine*	*Galactosamine*	*Hexose*	*Hexosamine*
Fresh Calf Skin	0.2	0.4	0.3	0.03	0.1
Protein extracted at pH 3.7					
Precipitated	0.1	0.1		0.02	0.01
Not Precipitated	3.2	11.7	5.4	0.5	2.7
Protein extracted at pH 2.8					
Not precipitated	18.6	129.8 (probably nearly all glucosamine)		3.3	23.2
Protein extracted by alkali and precipitated by ethanol	0.3	14.1	22.9	0.05	6.1
Extracted Calf Skin Residue	0.03	0.2	0.1	0.01	0.06

The amino-acid composition of the alkali-soluble fraction was also obviously different from that of collagen, though the presence of some hydroxyproline suggests that it contains a small amount of collagen, about 5 per cent, in the case of the particular sample examined. Even when allowance is made for the presence of this collagen, the arginine, leucine, isoleucine, tyrosine, proline, glutamic and aspartic acid contents are higher than those of the acid-soluble fractions. Appreciable amounts of ninhydrin reacting material were eluted from the columns in positions corresponding to glucosamine and galactosamine, and direct determination of hexosamine on the same hydrolysate indicated the presence of an amount corresponding to the two peaks.

The hexosamine and hexose contents of the various fractions were determined independently after milder conditions of hydrolysis with

2 N hydrochloric acid for 16 hours at 105° C. The hexosamines and hexoses were separated from the amino acids on an ion exchange resin (Dowex 50) and determined by modifications of the Elson and Morgan and Anthrone methods respectively (see Moss, 1955).

The results obtained are given in Table V expressed both as gm. per 100 gm. nitrogen or 100 gm. protein. In making the latter calculation, the nitrogen contents of the non-collagenous protein fractions were taken as 16.0 per cent and allowance was made for hexosamine nitrogen.

Both the hexose and hexosamine contents of the original skin, the acid-soluble collagen and the final extracted residue are low, and the greater part of the carbohydrate derivatives in skin appear to be associated with the non-collagenous fractions, particularly that extracted with the dilute acetic acid. It must also be borne in mind that appreciable amounts of hexoses and hexosamines may have been lost by diffusion through the cellulose membranes during dialysis and concentration. Except in the alkali-soluble fraction, the glucosamine content was greater than the galactosamine content. Hexuronic acids were not detected in the hydrolysates of any of these fractions, but as these substances are readily destroyed by acid this is no proof of their absence. If hexuronic acids equivalent to the hexosamine were present in the acetic acid-soluble fraction, however, they would almost certainly have been detected. Unfortunately there was insufficient material available to make a direct determination of uronic acids.

Extraction with Dilute Alkali

Some further experiments were carried out on the extraction of alkali-soluble protein. In these the whole thickness of the calf skin was used. The skin was closely shaved to reduce complications due to the solution of keratinous material, cut into pieces about 1 cm. in area and given a preliminary extraction with six changes of 0.1 M phosphate buffer, pH 9.0. Samples of the skin were then extracted with sodium hydroxide or calcium hydroxide solution at pH 12.5 at three different temperatures, 4°, 20° and 30° C. The total-nitrogen, ammonia-nitrogen, hydroxyproline-nitrogen, and tyrosine-nitrogen of the solutions were determined (see Table VI).

As before, the hydroxyproline values indicate that it is mainly a non-collagenous protein which is extracted. The amount of collagen dissolved increases with temperature to a greater extent than does

TABLE VI

ALKALINE EXTRACTION OF CALF SKIN. NITROGEN EXTRACTED AS A PER CENT TOTAL-NITROGEN OF PHOSPHATE-EXTRACTED SKIN

	Total-N	Tyrosine-N	Hydroxy-proline-N	Collagen-N
Sodium Hydroxide pH 12.5				
4° C.	4.4	0.11	0.01	0.1
20° C.	7.6	0.18	0.02	0.2
30° C.	9.2	0.19	0.06	0.7
Calcium Hydroxide pH 12.5				
4° C.	2.9	0.07	0.01	0.1
20° C.	5.9	0.17	0.03	0.4
30° C.	14.0	0.32	0.4	4.8

the non-collagenous protein, especially in the calcium hydroxide solutions. Increase in pH also increases the solution of collagen to a greater extent than that of the non-collagenous protein.

DISCUSSION

On the basis of this investigation it is suggested that the citrate-soluble collagen obtained after extensive purification by solution and reprecipitation approaches in composition that of the pure collagen protein, and that the various collagenous fractions obtained all consist basically of this protein, the small differences in composition between them being due to their association with varying amounts of non-collagenous type proteins.

Whether these non-collagenous proteins are derived from extraneous sources such as the plasma proteins, muscle fibres, cells, etc., or whether they represent an integral part of the collagen fibres, is difficult to decide. Possible sources of non-collagenous proteins in skin are the tissue fluids, blood vessels, muscle fibres, cellular tissue, elastic tissue and ground substance. The bulk of the cellular tissue, blood vessels, muscle fibres and elastic tissue are present in the outer layer of the skin, which was removed prior to extraction, but small amounts may be still present together with some tissue fluid proteins not removed completely by the phosphate extraction. The elastin of the elastic tissue and blood vessels is unlikely to be affected by the dilute acid and alkaline solutions used for extraction and will, therefore, be located in the extracted residue. It has been shown histologically that the cells remain intact during the citrate buffer

and acetic acid extractions but largely disappear during the alkaline extraction. Their contribution to these extracts is probably small compared with the total amount of protein extracted at this stage.

The non-collagenous protein fraction isolated from the citrate extracts is the most likely to contain protein derived from the tissue fluids, blood vessels and muscle fibres and it is possible that it represents small amounts of protein from a number of different sources rather than any specific protein. It has been found in experiments now in progress that preliminary alkaline extraction at 12.3 to 12.5 reduces this fraction to negligible proportions.

The acetic acid-soluble fraction differs in a number of respects from the corresponding citrate-soluble fraction, particularly in the large amounts of glucosamine and hexoses associated with it. This suggests that it is derived from a different less soluble source. There were, however, considerable losses of nitrogen-containing material during dialysis and concentration, and without a knowledge of the composition of this material, the full extent of the difference between these fractions cannot be determined. Preliminary extraction with alkali does not reduce this fraction and its solution appears to be contingent on the solution of collagen also. In the acetic and extracts there was no indication that the amount of protein was decreasing in successive extracts, and the results suggested that the fibres as a whole were very slowly going into solution. Judging from its hydroxyproline content the final extracted residue still contained some non-collagenous protein and it is possible that the non-collagenous protein isolated from the acetic acid extracts represents the soluble form of some of this protein. Attempts by Harkness, Marko, Muir and Neuberger (1954) to remove what is presumably a similar constituent from gelatin obtained from rabbit skin were only partially successful, so that it appears to be very closely associated with the collagen protein. As discussed earlier at least part of the glucosamine associated with this fraction is probably present as non-uronic acid containing polysaccharide.

The non-collagenous protein extracted by alkali probably comes mainly from the ground or cementing substance. Between 2 and 10 per cent of this non-collagenous protein can be extracted from calf skin, depending on the pH and the temperature of the extraction, and probably on the age of the animal. The removal of this fraction appears to run parallel with decrease in the cohesion of the skin, since

long treatment in alkaline solutions lowers the shrinkage temperature and facilitates the conversion of collagen to gelatin. The protein of this fraction was associated with relatively large amounts of both glucosamine and galactosamine. Unfortunately there was insufficient material available for direct determination of uronic acids without hydrolysis, and it is not certain to what extent these are present, and hence what proportion of the hexosamines are derived from hyaluronic acid and chondroitin sulphate. In view of the observations of Consden *et al.* (1953) and Consden and Bird (1954), regarding the presence of a non-uronic acid containing polysaccharide in alkaline extracts of connective tissue and skin, it seems probable that at least some of the hexosamine is also present in this form. It may be noted, however, that the hexosamine/hexose ratio is very much higher than that reported by Consden *et al.* (1954) for connective tissue.

It is considered that the evidence as a whole is in favour of the presence of two non-collagenous type proteins in collagenous tissue, as distinct from those of the tissue fluids, muscle fibres, etc. One of these, the greater in amount, is soluble in alkali and probably represents the protein of the ground substance, while the other is more closely associated with the collagen in the fibres. The amounts of these constituents probably vary with the nature of the tissue and with the age of the animal. Their influence on the stability of the tissue appears to be dependent not only on the amounts present but also on its state of organization. On the one hand removal of the alkali-soluble material increases solubility, while on the other hand decrease in the amounts of non-collagenous protein with age is accompanied by decrease in swelling and decrease in solubility (Banfield, 1952). This decrease in solubility may be due to closer association of the non-collagenous protein-polysaccharide complex with the collagen with the result that the number of cross-links binding the molecular units together is increased.

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GROUP DISCUSSION

Dr. Grassmann said that his results agreed essentially with those of Dr. Bowes but that there were some differences which he attributed to differences in methods of purification of collagen and preparation of procollagen. He said that his method of histidine estimation was reliable but that results could vary from one preparation of collagen to another.

Dr. Bowes thought that this variation might be due to differences in the amount of non-collagenous protein of high histidine content being present. She thought that crystallization of the citrate-soluble collagen in the form of needles did not necessarily mean that all such contaminants had been removed.

Dr. Grassmann thought that the low hexose content estimated by Dr. Bowes might be due to retention of some hexose on the Dowex 50 column. He found that different methods of hexose estimation gave good agreement. He commented on the quantitative nitrogen recovery but low recovery (95 per cent) by weight of amino acids which he had observed in collagen and procollagen and which Dr. Bowes had observed in procollagen.

Dr. D. S. Jackson said that when he crystallized procollagen it invariably had a higher hexosamine content than when it was precipitated with salt, when the hexosamine was invariably left behind in the supernatent.

Dr. Gross said that the electron microscope is useful in detecting small amounts of contaminant in procollagen precipitates by showing the presence of 'long spacing' fibrils. He said that when hexoses and hexosamines were estimated directly on the tissue, without hydrolysis (other than that in the reaction), by the orcinol method, higher and perhaps more reliable values were obtained than if the tissue was hydrolysed.

Dr. Bowes said that she had observed this effect with citrate-soluble collagen but with collagen identical results were obtained, whether the tissue had been hydrolysed first or not.

Dr. Neuberger suggested that the low nitrogen recovery in Dr. Bowes's analyses might be due to the formation of small amounts of diketopiperazines of hydroxyproline and of proline such as those which had been found by Dakin in hydrolysates of gelatin.

Dr. Grassmann said that in general diketopiperazines were less stable to acid or alkali than peptides.

Dr. Bowes said that she had found that the hydroxyproline values were unaffected by time of hydrolysis in the range of 24 hours to 72 hours.

Dr. Neuberger said that apart from a difference in histidine content, the amino-acid analysis of rabbit collagen from his department was in good agreement with Dr. Bowes's analyses for hide.

Dr. Bowes thought that there were also differences in tyrosine and leucine, that is, in those amino acids probably present in relatively high concentrations in the non-collagenous component.

Referring again to the unreliability of methods for determining sugars in connective tissue, Dr. Meyer said that the orcein method might give different values to the anthrone method for four reasons:

(1) the method depends on the standard used;
(2) it depends on the amount of browning;
(3) it depends on the type of sugar;
(4) it depends on the type of linkage.

He quoted the apparent difference in hexose content of heparin and disulphated heparin as determined by the orcein method. The results for the former were 2.5 to 3 times that to be expected from an amount equivalent to the uronic acid content as determined by decarboxylation. For the desulphated heparin, the amount of hexose determined was equivalent to the uronic acid.

Dr. Bowes confirmed Dr. Meyer's suspicion that the values might be unreliable. The amount of hexosamine estimated to be present in the acetic acid-soluble fraction was probably greater than the amount of hexosamine present in the original skin sample indicating that one at least of the determinations was unreliable.

Dr. Orekhovitch said that if large differences in tyrosine and histidine contents of the different fractions were due to a contaminant, one would expect differences in the contents of the other amino acids, which were not in fact observed.

Dr. Bowes pointed out that there was some loss of amino acids during the dialysis and that one should not, therefore, attempt to make too detailed a comparison of the amino-acid compositions of the different fractions and draw conclusions from this.

Dr. Orekhovitch thought that the differences between the fractions might be due to the presence of peptides rich in these amino acids (tyrosine and histidine).

Dr. Bowes sa d that small peptides would probably have been removed during dialysis, but it was possible that these amino acids might be present in large peptides which had been split off from the collagen. If such were the case the hydroxyproline must be concentrated in the remaining part of the chain, and although Dr. Grassmann's work indicated that a number of hydroxyproline residues are located near one another, it seemed unlikely that a very large peptide of the composition required by the analysis would be split off from collagen.

Dr. Orekhovitch said the peptides might be weakly bound to proteins and, therefore, not separated by dialysis. For example, serum albumin

adsorbs amino acids strongly, and they can only be separated by repeated fractional recrystallization.

Dr. Bowes said that the fractions were probably not homogeneous, and apart from saying that one or possibly two relatively large peptides or protein fractions of a non-collagenous nature had been isolated from skin, she did not think at this stage it was justifiable to draw further conclusions from the analysis of these fractions.

Dr. Grassmann said that if one avoided the use of concentrated strong acids in hydrolysis, the amino acids in the hydrolysate could be separated as their DNP derivatives from hexoses and DNP amino sugars on an ion exchange column.

Dr. Meyer thought that in the materials with which they were concerned, even hydrolysis with 1 N acid at 100° for 8 hours (Dr. Grassmann's hydrolysis conditions), some destruction of sugars, particularly uronic acids, might occur.

Dr. Grassmann replied that, except for uronic acids which he had not been concerned with he had found these conditions reliable. Even after hydrolysis for 20 hours, the amount of hexose estimated did not fall.

Dr. Gross thought that a qualitative examination of the hydrolysate should be done first so that one could choose appropriate standards for subsequent quantitative measurements. Using synthetic mixtures of carbohydrate and amino acids, he had found a gradual loss of hexoses as the mixture was hydrolysed with 2 N acid for increasing times and suggested that it might be due to impurity of the hydrochloric acid he had used. He thought that, for comparative purposes, more reliable results could be obtained by measuring the orcein colour directly by Friedman's method.

Dr. Neuberger pointed out that more ammonia was obtained in the Moore and Stein analysis of the acetic acid-soluble collagen than one would expect from the sum of the aspartic and glutamic acid content. He suggested that ammonia might arise from some source other than amide groups and asked if Dr. Bowes had determined amide nitrogen by the standard method of hydrolysis with 2 N acid for three hours.

Dr. Bowes said that some ammonia might have come from decomposition of hexosamine but that no direct amide nitrogen determinations had been done owing to shortage of material.

Dr. Consden said he had obtained results similar to those of Dr. Bowes by extracting washed human subcutaneous tissue with alkali. The protein extracted was largely non-collagen since it contained tryptophan, and was relatively rich in tyrosine and poor in hydroxyproline. The extract also contained 10–20 per cent carbohydrate, much of which was a mixture of sulphated acid polysaccharides. These were predominantly non-uronic acid containing and were comprised of hexosamine and hexose

(galactose or galactose+mannose). Similar protein and polysaccharide mixtures were liberated after digestion of the tissue with collagenase.

With dilute potassium hydroxide, somewhat less than half the original polysaccharide and non-collagen protein was extracted within a week. The remaining 50 per cent could not be extracted even after several months. It therefore appeared that in loose connective tissue the collagen fibres were closely associated with two types of non-collagen protein and acid polysaccharide, one of which was much more tightly bound than the other.

In reply to Dr. Consden, Dr. Bowes said she had no evidence that 'pure' collagen contained no tyrosine.

Dr. Grassmann said he had isolated a peptide from collagen which contained 42 amino acids. Each chain contained 2 tyrosine residues. The amount of peptide he had isolated was about 1.5 per cent of the collagen, and it might be present to the extent of 3 per cent. This indicated that there must be about 0.15 per cent tyrosine in collagen (see also, amino acid sequence).

Dr. D. S. Jackson said that his neutral salt-soluble collagen contained about the same amount of tyrosine. Hexosamine was absent and there was only a small amount of hexose and he thought that tyrosine was actually part of the collagen molecule.

Dr. Robb-Smith and Dr. Gillman thought that histological and histochemical techniques might be very useful in following the changes in tissues as they passed through the various extraction procedures.

Dr. Bowes said that preliminary work on these lines had been done. Staining with haem-alum indicated that the fibroblasts were apparently intact up to the end of acetic acid extraction, but disappeared during the alkaline extraction.

PROCOLLAGENS AS BIOLOGICAL PRECURSORS OF COLLAGEN AND THE PHYSICO-CHEMICAL NATURE OF THESE PROTEINS

V. N. OREKHOVITCH AND V. O. SHPIKITER

In our previously published work we made a hypothesis on the transformation of procollagen into collagen. A careful study of similar data published during recent years by other laboratories and their comparison with our data tends to confirm our original ideas concerning the biological role of the procollagens.

In this paper we shall give the results of our research concerning procollagens and, in the first place, their properties and their modes of transformation.

We shall begin by giving the results of the studies concerning the physico-chemical nature of procollagens and also all data concerning the molecular weight and the dimensions of the particles of these proteins. For some time it was considered that the molecular weight of procollagen isolated by the method used in our laboratory (extraction by an acid-buffer solution of citrate followed by dialysis against tap water) is 70,000 and the length of the molecules 380 Å (Bresler, Finogenov and Frenkel, 1950). Later, however, it was seen that these values corresponded not to native procollagen but to its thermal denaturation products, as the procollagen solutions were prepared at about 40° C. We now know that during such treatment the procollagen particles break down. In connection with this we started in 1954 various researches on the molecular weight of native procollagen (Orekhovitch and Shpikiter, 1955).

With this in view, we studied sedimentation, diffusion and viscosity of procollagen in citrate buffer solutions with a pH of 3.6 containing 1 per cent of calcium chloride or 0.5 M urea. The coefficients of sedimentation were measured for a series of concentrations. By extrapolation to infinite dilution we found that $s = 3.05$–3.25 Svedberg units. For the diffusion constant we have obtained $D = 0.35$–$0.40 . 10^{-7}$ cm.^{2}/sec., and one of these determinations was made with the Tsetkov polarizing interferometer (Tzvetkov, 1951) with a concentration of protein of 0.02 per cent, that is to say a concentra-

tion which allows one to consider the particles in solution as kinetically independent. Viscosity was measured by the U-shaped capillary viscosimeter with different shear gradients (from 2500 to 100 seconds^{-1}); our data enabled us to calculate the value of intrinsic viscosity $[\eta] = \eta_{sp}/c$, with $c \rightarrow 0$ (η_{sp}-extrapolated to $q = 0$ and with c-g/100 ml.) which is found to equal 16-17. The molecular weight calculated from these data corresponded to 700,000 and the length of the molecule to nearly 7000 Å. The difficulties we met in our research on procollagen solutions by physico-chemical methods do not allow us to consider these data as exact. It is more accurate to speak of an order of magnitude. It should be said that similar results were obtained by the Japanese worker Noda (1955) for acid soluble collagen similar to procollagen. This worker obtained $s = 3.5 \cdot 10^{-13}$; $D = 0.5 \cdot 10^{-7}$ cm.^{2}/sec. and calculated $M = 710{,}000$ and length of the particles nearly 5000 Å. For ichthyocol (very similar to procollagen) extracted by a citrate buffer solution, Gallop (1955) obtained $s = 2.85 \cdot 10^{-13}$, $[\eta] = 13.2$, the molecular weight determined by light scattering $1.7 \cdot 10^6$, and the length of particles = 4000 Å.

Boedtker and Doty (1955) have also studied the citrate extract of ichthyocol and obtained $s = 2.85 \cdot 10^{-13}$, $[\eta] = 11.5$. On the basis of these data, and assuming a hydration of 34 per cent and a high asymmetry, these workers obtained a molecular weight of 300,000, remarkably similar to the results obtained by the study of osmotic pressure (300,000) and light scattering (340,000). The length of the molecule was found to be 3000 Å by flow-birefringence. The similarity of the average molecular weights obtained from osmotic pressure and light scattering measurements indicates that this protein is very homogeneous. In addition, several papers were published on the determination of the molecular weight of procollagen proteins by the method of light scattering, and values of the order of 10^6-10^7 were obtained. However, one should accept with great caution values obtained by the light scattering method because of the great difficulty of freeing the solution from dust, the presence of which alters the results considerably. For the time being we shall not analyse these data for the determination of molecular weight. It is important to stress that the molecular weight and the length of the molecule of procollagen are fairly high.

It should be said that for procollagen and similar proteins, the constants of sedimentation $s = 3 \cdot 10^{-13}$ and values of intrinsic visco-

sity $[\eta] = 15$ are very characteristic, and it is for this reason that these data may be used for the identification of procollagen.

To conclude this part, concerning the physico-chemical nature of procollagen, we shall quote a few of our results relating to its macro-structure. When solutions of procollagen are heated at a certain temperature (according to the pH of the milieu) one observes a considerable lowering of viscosity (at pH = 4 the temperature is around 37° C.; at pH 2 the temperature is around 30-32° C.; at pH 6 around 40° C.); urea in a concentration of 3 M at pH 4 lowers the temperature at which viscosity drops to 33° C. and at a concentration of 6 M the temperature at which viscosity drops falls to 23° C. The same lowering of viscosity was seen with concentrated solutions of salts (for example with 5 M KCNS, KI, $CaCl_2$, etc.). The same action occurs with hydrogen peroxide at a concentration of approximately 20 per cent. In all these cases the drop in the viscosity is accompanied by irreversible modifications of the procollagen molecules, viz. breakdown into simpler components.

The products of disintegration differ considerably from the native procollagen. Contrary to it, these products give thixotropic gels, are not precipitated by 30 per cent acetone or by 4 per cent sodium chloride, and by their properties are related to gelatin.

During ultracentrifugation, after heating the solution of procollagen (citrate-buffer solution at pH 4 containing 3 M urea at 30° C.) for 10 minutes, two components are detected with sedimentation constants $s = 2.4 . 10^{-13}$ and $s = 3.5 . 10^{-13}$. We obtained similar results by heating a suspension of procollagen in a phosphate buffer solution at pH 8 at 70° C. during 10 minutes with subsequent addition of a solution of 2 M of KCNS to a final concentration of 1 M (see Fig. 1*a*) or by dissolving the procollagen at room temperature in a buffer solution containing 5 M of KCNS with subsequent addition of buffer until a concentration of 1 M of KCNS was reached (see Fig. 1*b*). The heating of the suspension of procollagen and the action of KCNS (by adding to the heated solution dry KCNS to a concentration of 5 M with subsequent dilution of the solution to 1 M KCNS) gives a similar picture, as can be seen from Fig. 1*c*; the procollagen breaks down into two components. These results lead us to think that the compounds detected by ultracentrifugation are constituent elements of the molecules of native procollagen. It is interesting to note that the character of the disintegration products of the molecule of procollagen is similar and does not

depend on the nature of the substances which act upon it. This is to say that the same results are obtained by the action of heat, of urea, or of high concentrations of salts. At the present time, we are continuing these studies, and we intend to isolate these components.

We shall now consider the data which confirm our hypothesis that procollagen is a biological precursor of collagen.

First, in favour of this hypothesis, we have the data relating to quantitative variations in the content of procollagen in skin with age. In young animals, the amount of procollagen is fairly high, and as the organism becomes older it diminishes greatly.

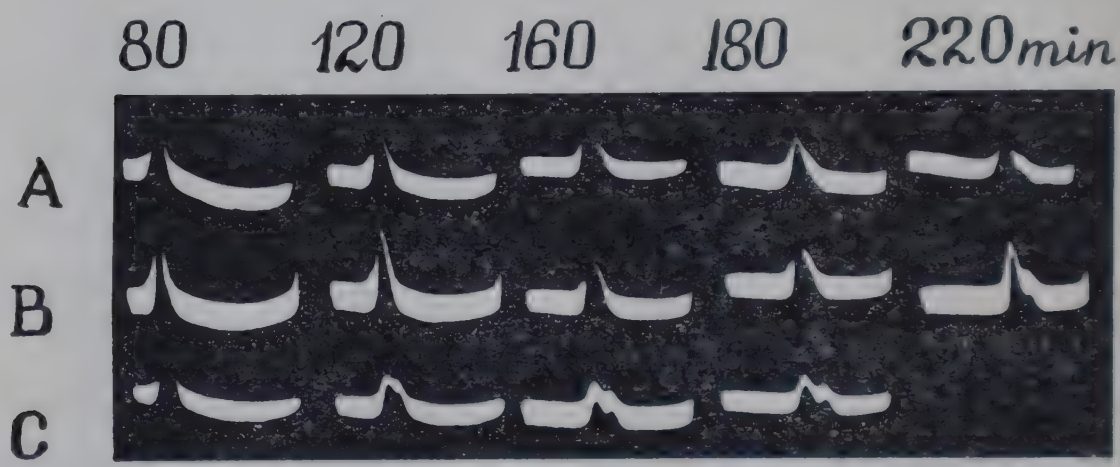


Fig. 1

Sedimentational diagrams of procollagen breakdown products. Svedberg ultracentrifuge, 56,000 r.p.m., rotor temperature 28-30° C. Solvent 1/15th M phosphate buffer, pH 8+ 1 M KCNS.

A, heated at 70° C. for 10 min.

B, dissolved in 5 M KCNS.

C, heated at 70° C. for 10 min., then treated with 5 M KCNS.

Second, in certain pathological states, for example in cases of vitamin C deficiency, one notes an arrest in the formation of new collagen fibres. Assuming that the inhibition of collagen formation in the organism is determined to a certain extent by the slowing of the synthesis of procollagen, we have studied in our laboratory the quantity of this protein in guinea-pigs suffering from scurvy. We have established that in healthy animals the skin contains 10 per cent of procollagen (in relation to the weight of desiccated and defatted skin) whereas in scorbutic animals the amount is 5 per cent. The amount of collagen in the skin of both groups of animals remains

constant when compared to the quantity of collagen in the skin of healthy animals at the time of the onset of scurvy. More convincing data on the inhibition of the synthesis of procollagen in the skin of animals suffering from scurvy were obtained in experiments with radioisotopes. We injected under the skin of healthy and affected guinea-pigs glycine containing radioactive carbon on the carboxyl group. After a variable lapse of time following the injection of labelled glycine the animals were sacrificed and the procollagen and collagen (gelatin) isolated from their skin. The radioactivity of these proteins was determined. In Table I we give the results and it can

TABLE I

RATE OF INCORPORATION OF ^{14}C GLYCINE IN THE PROCOLLAGEN AND COLLAGEN OF HEALTHY AND SCORBUTIC GUINEA-PIGS (IMP./MIN. FOR 10 MG. OF PROTEIN)

Substance	*Time elapsed after injection of the amino acid (hours)*							
	3	6	10	18	24	120	168	384
Procollagen								
Scorbutic animals	3	6	4	2	1	0	3	0
Healthy animals	45	134	140	192	190	122	89	70
Collagen								
Scorbutic animals	1	1	0	0	2	—	3	3
Healthy animals	9	12	13	12	16	—	23	25

be seen that ^{14}C glycine is not found in the procollagen of animals suffering from scurvy, that is to say that the synthesis of this protein is completely inhibited, which corresponds to an arrest in the formation of collagen.

Third, the study of the rate of incorporation of radioactive glycine in the procollagen and collagen of healthy animals following at various intervals after the injection of this amino acid confirms our concept that procollagen is a precursor of collagen. As can be seen from Table II, the greatest radioactivity of procollagen is found after 12-24 hours after the injection of radioactive glycine in the animals.

In the following days, radioactivity of procollagen diminishes rapidly, and on the fortieth day it reaches values lower than those for collagen which are found 3 hours after the injection of radioactive glycine. For collagen it is the opposite process which is characteristic.

The longer the time following injection of radioactive glycine, the

TABLE II

RATE OF INCORPORATION OF ^{14}C GLYCINE IN THE PROCOLLAGEN AND COLLAGEN OF GUINEA-PIGS (IMP./MIN. FOR 10 MG. OF PROTEIN) AT VARIOUS TIMES AFTER THE INJECTION OF AMINO ACID

Observed object	*Time elapsed after injection of amino acid (in hours)* 3	6	12	24	96	480	840	840	960	960	960	960
Procollagen												
1st extraction	71	119	130	157	97	84	32	18	7	8	6	14
2nd "	62	114	110	119	75	—	44	37	39	20	12	38
3rd "	40	79	84	84	52	52	40	41	41	44	27	40
4th "	50	60	64	64	42	44	52	43	71	38	33	41
5th "	35	65	60	64	53	—	55	35	70	—	35	33
6th "	27	32	38	48	32	38	32	31	54	29	30	35
Collagen	12	17	22	26	30	40	43	39	80	53	44	44

higher the radioactivity of the collagen isolated from these animals. After 3 hours, the radioactivity of collagen corresponds to 12 imp./min. for 10 mg. of protein; 40 days after, it corresponds to 80 imp./min., that is nearly seven times more. If we compare these data with the radioactivity of the first extraction of procollagen and collagen, one is struck by the following fact: 24 hours after injection in the organism of radioactive glycine, the radioactivity of procollagen begins to decrease, being practically nil around the fortieth day, whereas for collagen it is towards the fortieth day that it is maximum. At that time, the radioactivity of collagen is approximately twelve times greater than that of procollagen. If we take into account the fact that the incorporation of radioactive glycine in these proteins takes place only at the time of their formation, this phenomenon can be explained in the following fashion. In the first 24 hours after the injection of radioactive glycine, a synthesis of procollagen takes place with the utilization of ^{14}C glycine, and it is for that reason that the protein fraction obtained from the first extraction contains a radioactive procollagen. On the second, third and other extractions, it is principally the protein substances synthesized before the injection of ^{14}C glycine which go into solution and it is for that reason that the fractions of procollagen are less radioactive. The collagen is not very radioactive either at that time as we have observed in reality. As procollagen transforms itself into collagen by passing through intermediary protein substances, the radioactivity of these substances and of collagen increases. After a certain time the procollagen synthesized at the time of the greatest concentration of

radioactive glycine in the organism will transform itself into collagen which will enrich itself in radioactive carbon. At that time the original amount of radioactive carbon is already eliminated from the organism and the procollagen newly synthesized contains very little radioactive carbon. The result is that the radioactivity of collagen will considerably exceed that of procollagen. That is what we have seen in our experiments.

At the present time, a great amount of interest is aroused by the collagen-form proteins extracted from connective tissue by slightly alkaline or neutral saline solutions (the so-called alkali-soluble collagen, tropocollagen, etc.). Sometimes these are taken for real precursors of collagen whereas the citrate-soluble collagen (procollagen according to our terminology) is sometimes taken for collagen. We believe it is important to discuss the relationship of these proteins and to clarify as far as possible the question of the precursors of collagen.

At the time we published our results concerning the study of the metabolic activity in procollagens with the use of ^{14}C glycine, Professor Neuberger was publishing results similar to our own. Subsequently, Professor Neuberger and his co-workers (Harkness *et al.*, 1954) expressed the opinion that other precursors of collagen may exist. Among other points, he relates to these other predecessors the alkaline-soluble fractions of collagen. Gross, Highberger and Schmitt (1954), on the basis of electron microscopic research on various structural forms of collagen precipitated under various conditions from tissue extracts, made the hypothesis that primary structural units having a length of 1500-3000 Å are present and that their corresponding length was related to the cross striation of the fibres. These hypothetical particles were called tropocollagen, and it was assumed that they could transform themselves into collagen. It was shown subsequently that collagen could be extracted by neutral saline solutions. It was supposed that this tropocollagen passing into solution exists in the ground substance of connective tissue whereas the 'procollagen of Orekhovitch' comes from newly formed collagen fibrils (Gross *et al.*, 1955). This supposition was based on the fact, obtained by electron microscopy, that collagen fibres in weak alkaline solutions and neutral solutions are not modified whereas the structural organization of these fibres is greatly altered by extraction with an acid buffer solution.

The following scheme, suggested by Grassmann (1955), thus ensues:

Tropocollagen – Procollagen – Collagen.

We present this scheme, for greater clarity, in tabular form:

TABLE III

Suggested names	*Characteristics*
Tropocollagen	Extracted by saline solutions either weakly alkaline, neutral or acid. Gives a periodic cross striation of fibres of 2,000 Å. Solutions are stable at 4 to 37° C. Precipitate formed at 37° C. Metabolically very active. Present in ground sub stance of connective tissue.
Procollagen	Extracted by acid solutions and by acid buffer solutions. Precipitated by neutral and alkaline solutions. Gives a periodic cross striation of 650 Å. Deaggregates when heated to 37° C. in acid solutions. Metabolically less active. Present in newly formed collagen fibres.
Collagen	'Condensed' fibres. Insoluble in solutions weakly alkaline or neutral. Gives cross periodic striation of 650 Å. Very little metabolic activity. Constitutes the principal mass of collagen fibres.

In the discussion of this scheme, it is essential to quote the data which characterize the physico-chemical nature of tropocollagen and compare them with those of procollagen. We have isolated by the method of Neuberger an alkali-soluble collagen and have compared it with the procollagen extracted by our method. We have been able, first, to obtain this protein in needle 'crystals' identical to those of procollagen. We have established that alkali-soluble collagen contains 27 per cent glycine and 10.2 per cent hydroxyproline, and procollagen respectively 26 per cent and 10 per cent. For alkali-soluble collagen in 0.1 M citrate buffer solution at pH 4 containing 1 per cent of $CaCl_2$ we obtained $s = 3.1 . 10^{-13}$ and $[\eta] = 13.0$ (for procollagen in these conditions $s = 3.05 . 10^{-13}$ and $[\eta] = 17.5$). We may conclude that these two proteins, by their physico-chemical properties, are practically identical.

We may add that Jackson and Fessler (1955) found for collagen soluble in saline solution with a neutral pH a value of $s = 3.23 \times 10^{-13}$, a molecular weight of the order of 10^6 and a length of particles by birefringence corresponding to 4000 Å. If we take into account

the errors of the methods and the differing conditions of these experiments we may say that the physico-chemical characteristics of tropocollagen and of procollagen correspond very closely and, for this reason, the particles of these proteins do not differ fundamentally from each other and have the same physico-chemical nature.

With regard to solubility, the problem is not finally settled. We have seen that procollagen obtained in the second and third extractions with citrate-buffer solution could be dissolved in 0.1 M sodium citrate (at pH 5) in considerable quantity and that, if an alkaline solution was added until pH 7 was reached, the solution was stable. However, when it is heated up to 37° C., a precipitate of protein is formed, that is to say that procollagen behaves like tropocollagen. We, therefore, believe that the solubility of tropocollagen with a neutral pH or a weakly alkaline pH does not prove that its nature is fundamentally different from that of procollagen.

The periodicity of cross striation is not a good criterion of the nature of these primary particles. This is particularly clear in the experiments of Schmitt and others (1953) on the reciprocal transformation of various structural forms of collagens precipitated under different conditions.

With regard to the metabolic activity of tropocollagen we must point out that in extracting finely minced skin tissue with a citrate buffer solution (without prior treatment with phosphate) we have obtained in the first extract a tropocollagen with a greater metabolic activity.

GROUP DISCUSSION

Dr. D. S. Jackson suggested that the differences of opinion between himself and Dr. Neuberger on the one hand and Dr. Orekhovitch on the other, were not so much real differences as ones of language. He suggested that Dr. Orekhovitch's extraction procedures produced both neutral-extracted collagen and citrate-extracted collagen and that, although they were both substantially referring to the same product, theirs was the more representative precursor since the properties of their particular preparation could be accounted for on the assumption that it was the true precursor of collagen fibres.

Dr. Orekhovitch explained that in his method the citrate extraction was preceded by extraction with phosphate or other neutral solution. He felt that such preliminary extraction would remove all extraneous

material leaving the procollagen to be extracted by the subsequent citrate solution.

Dr. D. S. Jackson asked how Dr. Orekhovitch explained the fact that in the neutral extract there was a collagen component which took up radioactive ^{14}C glycine at a greater rate than his citrate extract.

Dr. Orekhovitch stated that the high activity of the fraction extracted by neutral solution was due to the presence of the serum proteins alone since it contained little collagenous material.

Dr. D. S. Jackson replied that in their opinion this could not be substantiated since the collagen component was highly purified, and the rate of uptake of ^{14}C glycine into the non-collagenous fraction was different from its rate of incorporation into the collagen extracted at the same time.

Dr. Orekhovitch stated that he had seen no numerical data on this matter, whereupon Dr. Neuberger quoted experiments from which it could be shown that the specific activity of radioactive glycine isolated from the alkali-soluble fraction of collagen after the administration of radioactive glycine to a young rabbit was either identical or greater than in the serum protein. If Dr. Orekhovitch's observations were correct, one would expect a hundred times greater concentration in the serum proteins than in the alkali-soluble collagen and this was not observed. The only explanation is the existence of a protein with an exceptionally high specific activity.

Dr. Gross drew attention to the fact that Dr. Orekhovitch had stated that the neutral salt extract contained little collagenous material. He pointed out that, if extracts were made from the tissues of rapidly growing animals, using a buffer tissue ratio of 2 : 1, the extract contained as much as 0.3 per cent collagen. Starvation, accompanied by cessation of growth, however, caused a marked drop in the amount of collagen extracted, and it could be that Dr. Orekhovitch's results were due to lack of growth in his animals. On the question of terminology, he felt that Dr. Orekhovitch's use of the word 'tropocollagen' needed clarification. The term tropocollagen was not intended to imply precursor or source of a collagen unit but was to be taken as referring to a building block. Tropocollagen can be obtained by extraction of material which has never been incorporated into the fibre but it can also be taken as representing material which could be extracted from the fibre itself. Acid-extracted collagen, however, must be considered as differing in source from salt-extracted collagen since electron-microscopical examination of the fibres after acid extraction showed them to be considerably degraded. In other words, the acid-soluble collagen must be assumed to have come from the fibres themselves. This is further evidence in favour of the contention that the neutral salt-soluble collagen is the actual precursor of the fibre.

Dr. Orekhovitch answered, firstly, that all his animals were in a good

nutritional state, hence starvation could not be taken as the reason for his failure to find procollagen in alkaline extracts. The morphological characteristics of the fibrils should not be confused with their chemical analysis, and hence procollagen should be regarded as a chemical rather than a structural precursor. Dr. Orekhovitch then asked if tropocollagen could be classed as a micromolecule, to which Dr. Gross replied no, it was 2000-3000 Å long by 15 Å wide and represents at the present time the smallest collagen unit that can be converted directly into a fibril: any smaller particle being probably a breakdown product which cannot be directly built into a fibril.

In reply to a question by Dr. Orekhovitch, Dr. Gross stated that tropocollagen could be compared with the whole of the insulin molecule of molecular weight 34,000, and not to the 6000 molecular weight portion thereof. He added that he was not sure whether globular protein molecules should be directly compared with fibrous protein molecules.

Dr. Neuberger said that he regarded tropocollagen as comparable with the insulin monomer from which the Boston workers had shown the fibrous form of insulin to be built up.

Dr. Bear pointed out that native fibrous proteins, with possible exceptions such as elastin, are gradually being found to be like the soluble, crystallizable ones in being constructed of homogeneous particulate units (monomers). Collagen and feather keratin provide the cases best characterized to date. The monomers of the fibrous proteins are more asymmetric and often more difficult to disperse for physico-chemical characterization than are those of the soluble, crystallizable ones, which are generally described as globular.

Dr. Gross felt that the difference between the position in elastin and that in the collagen field was that, whereas the particles described by Dr. Partridge could not be rebuilt to make an elastin fibre, tropocollagen could. Any smaller particle which could be obtained could not be converted into a fibril and hence could not be regarded as the building block.

Dr. Orekhovitch inquired whether correlation could be made between this state of affairs and that occurring in the myosin and tropomyosin field. To which Dr. Gross replied that there was no evidence that tropomyosin could be converted into myosin, since, as Dr. Neuberger added, Bailey has shown that there is no close similarity between their respective amino-acid compositions.

Dr. Gross suggested that they intended the term 'tropocollagen' to be used in a generic sense, whereas 'procollagen' was more specific in that the material concerned had come from the fibril.

Dr. Bear remarked that there is little evidence as yet that the collagen molecules isolated by various extraction procedures are in fact significantly different chemically. For this reason, biochemists may be reluctant to

regard any one fraction as a precursor of the others, as indeed Professor Astbury stated in his opening remarks. However, as a means of following time sequences of events subsequent to the formation of collagen molecules by the cell, the various extracts may be useful. Depending on the methods used, events will be intercepted differently, but for some phenomena the general results with either neutral or acid-citrate extracts may be similar, since both represent fairly early stages in the long life history of collagen molecules.

DR. NEUBERGER agreed that isotope experiments in themselves do not prove that any substance is a precursor. All that one can say is that the results were compatible with the concept that the salt-soluble collagen is on the way to being formed into insoluble fibre. He also suggested that tropocollagen could be regarded as similar to the bricks from which a house can be built but in exactly the same way they could be regarded as the bricks obtained when the house was knocked down. Continuing this simile, DR. GRASSMANN suggested that the procollagen must represent newly laid bricks in which the mortar was not yet set since it could actually be extracted from the fibres themselves.

DR. GILLMAN inquired when 'stabilization' of the building units could be said to have occurred since he pointed out that scar tissue of considerable age could disintegrate if an animal bearing such old scars is rendered scorbutic. He suggested that isotope experiments on scar tissue might afford very useful data concerning the time taken for the incorporation of these apparently loosely bound portions of the fibre.

Concerning the question of solubility, DR. BEAR remarked that this term presupposes a definite and stable solid phase with which the dispersed molecules can be in equilibrium. The integration of collagen molecules into a fibril requires many adjustments, such as the formation of many intermolecular hydrogen bonds, and attainment of the most stable fibrillar structure may therefore require some time. Consequently, the past history of collagen fibrils, in the tissue or reconstituted from extracts, is important in determining the properties observed on a given occasion.

DR. HALL raised the question of the life expectation of collagen fibres, and DR. NEUBERGER indicated that this might be a very considerable period indeed.

DR. GILLMAN felt, however, that in certain of the tissues which he had mentioned, e.g. arteries, evidence indicated either a far shorter life span or a more rapid turnover than hitherto expected – especially in the presence of injury – whether metabolic or physical.

DR. NEUBERGER, however, stated that the very long life of the collagen fibre was indeed true of many tissues; but, of course, this applied only to animals in adequate nutritional state.

DR. D. S. JACKSON pointed out that studies in Manchester had shown

that, although collagen was removed rapidly from atrophying limbs, there was no evidence of a marked replacement of collagen during such experiments, as measured by the uptake of ^{14}C glycine.

DR. GILLMAN asked if any evidence was available from the comparison of rates of turnover of connective tissue components in a variety of sites. In answer to which DR. NEUBERGER gave figures for radioactive glycine incorporated into the collagen of rat's tail, bone, liver and skin. Although these figures were complicated by the fact that growth continued, the half-life of the fibres would appear to differ somewhat but to be in excess of 50 days.

DR. OREKHOVITCH stated that they had studied collagen from skin, liver, intestine and aorta and believed that there was finite value for the half-life, but that this value increased with age.

DR. GILLMAN drew the attention of the meeting to the fact that the tissues which had hitherto received greatest attention by chemists seemed to be those containing small quantities of reticulin and which would be expected to have fibres with a very long half-life. It seemed that the rate of fibre turnover, and especially of the related polysaccharides in vessels still merit attention by chemists.

THE STRUCTURE OF A CHONDROITIN SULPHATE COMPLEX FROM CARTILAGE

H. M. MUIR

Chondroitin sulphate was extracted from hog laryngeal cartilage with 10 per cent $CaCl_2$ by the method of Blix and Snellman (1945) and purified by precipitating from dilute solution twice with $Co(NH_3)_6Cl_3$ and twice with 5-aminoacridine at pH 4, the free polysaccharide being regenerated each time by shaking with cation exchange resins.

The substance appeared to be electrophoretically homogeneous and its aqueous solution had a high viscosity (0.5 per cent soln. η rel. = 6.1 at 25° C.). The analytical figures showed that there was 1.64 per cent of non-hexosamine nitrogen, equivalent to about 10 per cent of amino acids (Table I).

TABLE I

CHONDROITIN SULPHATE FROM HOG LARYNGEAL CARTILAGE

	Found (per cent)	*Found after papain treatment*	$C_{14}H_{19}O_{14}NSNa_2.4H_2O$
Hexosamine (Elson and Morgan)	29.18	31.5	31.1
Na. (flame)	6.62	7.15	8.01
SO_4. (Benzidine)	14.46	16.55	16.68
N. (Kjeldahl)	3.92	2.93	2.44
Non-hexosamine nitrogen by difference	1.64	0.47	—

The viscosity of the aqueous solution was unaffected by heating for 30 minutes at 100° C., but was quickly destroyed by weak alkali at room temperature, while even at pH 8.5 the viscosity began to fall slowly. No flocculation occurred either on heating or treatment with protein precipitants.

Crystalline proteolytic enzymes affected the viscosity to different degrees (Table II).

TABLE II

EFFECT OF ENZYMES ON VISCOSITY OF CHONDROITIN SULPHATE (1 PER CENT SOLUTION)

Enzyme (10 µg./ml.)	*Time after adding enzyme (minutes)*	*Per cent fall in viscosity*
Trypsin	100	24
,,	200	25.7
Chymotrypsin	160	17
Carboxypeptidase	120	6
Pepsin	100	48
Papain	10	80
,,	100	84

Trypsin, chymotrypsin and carboxypeptidase had comparatively little effect, although pepsin was more effective. In contrast activated papain caused the viscosity to fall by 80 per cent in 10 minutes. Inhibition by sulphydryl reagents showed that the effect was due to papain itself and not to any contaminating papaya lysozyme (Smith, Kimmel, Brown and Thompson, 1955). 4-6 per cent of dialysable hexosamine and sulphate appeared after papain treatment. Chondroitin sulphate which had been degraded by papain or by alkali could be chromatographed on paper with 45 per cent v/v n-propanol/55 per cent, 0.2 M boric acid and stained with toluidine blue. A compact spot of $R_f = 0.58$ was obtained, while the undegraded material remained at the origin. There appeared to be a correspondence between loss of viscosity and chromatographic behaviour, as the mobile spot was not produced either by heating or by those enzymes which did not markedly affect the viscosity. Pepsin had an intermediate effect and produced a mobile spot, a spot at the origin and intermediate streaks.

Two-way paper chromatography together with paper electrophoresis of the hydrolysed polysaccharide showed the presence of 12 amino acids. No sulphur containing amino acids could be detected and aromatic amino acids were scarce. Only a small amount of phenylalanine and a trace of tyrosine was found. No tryptophan could be identified by U.V. absorption of the polysaccharide. The amino acids present in largest quantity were glutamic and aspartic acids, serine, glycine, alanine, valine and leucine. Threonine and proline were present in smaller amounts and a small amount of lysine was identified by paper electrophoresis at pH 8.6. No hydro-

xyproline was detected by the Neuman and Logan procedure and no neutral sugars could be detected after mild acid hydrolysis by paper chromatography.

The papain digested product after prolonged dialysis was twice precipitated from dilute solution with 5-aminoacridine, converted to the sodium salt and finally precipitated with 70 per cent alcohol in the presence of sodium acetate. The analytical figures show a small residue of 0.47 per cent of non-hexosamine nitrogen (cf. Table I). Paper chromatography of the acid hydrolysate showed that papain had reduced the relative quantities of all the amino acids with the exception of serine which now stood out on the chromatogram. The papain digested material was treated overnight with 0.1 N alkali at room temperature, neutralized and dialysed, and the contents of the sac hydrolysed with acid. Paper chromatography of the hydrolysate showed that the residual serine had now been removed.

These results would suggest that the chondroitin sulphate which is relatively easily extracted from cartilage is a complex consisting largely of polysaccharide cemented by a small amount of peptide or protein. This complex is rapidly destroyed by papain leaving a polysaccharide which still appears to retain serine. This serine is easily removed by 0.1 N alkali and since the chondroitin sulphate complex also loses its viscosity in 0.1 N alkali, it is possible that the protein or peptide is attached to the polysaccharide through serine by an alkali labile bond.

GROUP DISCUSSION

Dr. Consden asked whether the strong spots which were visible on the chromatograms did not correspond with the bases; to which Dr. Muir replied that it was not possible to identify definitely any bases on the chromatogram. One of the spots concerned was galactosamine and the other was also a sugar, as yet unknown.

Dr. Partridge stated that by employing ion-exchange resin to remove the collagen, he had obtained a very similar protein polysaccharide complex from bovine nasal septa. The protein complex was stable to relatively high salt concentrations but could be broken by cold alkali or boiling dilute acetic acid. The analyses were similar to those of Dr. Muir but the protein did contain basic amino acids.

In answer to a further question by Dr. Partridge, Dr. Muir gave the

composition of her chromatographic solvent for C.S.A. as 45 per cent n-propanol, 55 per cent 0.2 M boric acid, and the sugars were stained with toluidine blue.

Dr. Meyer suggested that the basic spot might be chondrosin. Dr. Meyer also inquired whether any electrophoresis figures had been obtained, to which Dr. Muir replied that the material ran slightly differently from C.S.A.

Dr. Partridge gave similar results and suggested that part of the polysaccharide was bound and part free and that the two had similar electrophoretic mobilities. His material was extracted by potassium carbonate/potassium chloride mixture, the alkaline pH of which was commented on by Dr. Meyer.

Dr. Muir said that alkali brought about a lowering of viscosity, and because of this she had used the method of Blix and Snellman since it was the only method which she could find in the literature which made no use of alkali; and, in reply to a question by Dr. Neuberger, indicated that this was probably the reason why the majority of the material she had extracted occurred in the combined form whereas other workers, using other methods, apparently as gentle, obtained polysaccharide with very low nitrogen content.

Dr. Snellman pointed out the difficulty of securing complete separation from protein by the Sévag technique, and drew attention by analogy to the attempted separation of intact nucleo-protein.

Dr. Partridge felt that the very methods employed for the separation of the protein from the C.S.A. might also remove the mucoid material.

In answer to a question by Dr. Meyer, Dr. Muir stated that she did not think chondrosin would stand up to the hydrolytic conditions employed but the two sugar spots on her chromatogram both gave the Elson and Morgan reaction.

Dr. Meyer drew attention to the work of Schubert in which the extracted complex had an identical electrophoretic mobility to free C.S.A. and said that he felt this was a disturbing observation since one would have expected the free and bound polysaccharides to move at significantly different rates especially if the protein did contain free amino groups. He remembered that Mörner had claimed to extract a certain amount of C.S.A. with distilled water. They had often obtained nitrogen and hexosamine values which did not agree and felt that this was due to the fact that much hexosamine was destroyed during hydrolysis. On the other hand, decarboxylation could give a true uronic acid figure, and from this the total polysaccharide content could be calculated. However, by exceptionally mild methods they had obtained hexosamine preparations which were essentially consistent with the amount of C.S.A. present. The type of mild treatment which they had employed included

the Sévag technique and adsorption on Lloyd's reagent and ion-exchange resins.

Dr. Muir stated that instead of decarboxylation measurements she had estimated the uronic acid carboxyl groups by difference between the sodium and sulphate analyses.

In reply to a question by Dr. Gross, Dr. Muir stated that she could not find any tyrosine by ultraviolet absorption but agreed that it might have been destroyed during hydrolysis, especially in view of Dr. Gross's statement that tyrosine was considerably destroyed in the presence of large amounts of polysaccharides at acid pH.

Dr. Consden and Dr. Partridge drew attention to the difference between Dr. Muir's material and that prepared respectively by Schubert and by Partridge. The former found 18 per cent of tyrosine and the latter, in the protein extracted from the complex, a tyrosine content of 4 per cent.

In answer to a question by Dr. Meyer regarding the nature of the linkage between the polysaccharide and the protein, Dr. Muir stated that it was broken by decinormal alkali and hence one naturally thought in terms of an ester.

THE ARCHITECTURE OF THE COLLAGEN FIBRIL

R. Reed

The first model for the configuration of the polypeptide chains in collagen was proposed almost twenty years ago (Astbury, 1940). By integrating the features of the wide-angle X-ray diffraction pattern with the chemical and physical data such as were then known, it afforded a most useful basis for workers in the field of collagen structure. This original model was essentially a straight single polypeptide chain, so that the complete fibril was considered to be built by the parallel arrangement of this chain. In recent years, however, it has gradually been realized that the polyptepide chains in the collagen fibril do not follow a straight course; on the contrary, they appear to possess a helical configuration (Ramanchandran and Kartha, 1955; Ramanchandran, 1956; Rich and Crick, 1955; Cowan *et al.*, 1956). Thus workers in the field of X-ray diffraction now accept the notion of a helical configuration, the protofibrillar unit ('the thinnest filament which carried the essential chemical and configurational structure of collagen', Bear, 1952), being regarded as a coiled-coil system of three polypeptide chains.

It is pertinent, therefore, to discuss whether such a protofibrillar unit might lead to helical structures at higher levels of organization, such as are observed in the electron and in the ordinary light microscope. Most recent reviews of the architecture of the fibril (Bear, 1952; Grassmann, 1955), while accepting the helical structure of the protofibrillar unit, still account for the filaments and the complete fibril itself in terms of a parallel arrangement of this unit. Thus Fig. 1 shows the various levels of organization within the fibril as suggested by Bear (1952). Furthermore, the cross-banded appearance, which is a characteristic feature of the collagen fibril, has been accounted for in various ways, all based on an essentially parallel ordering of the protofibrils. Bear (1952), for example, considers that it is determined by the degree of perfection in which the protofibrils are organized together sideways. In the interband regions, the protofibrils are packed in almost perfect array, whilst in the band regions, the packing is less perfect. Other workers, however (Grassmann,

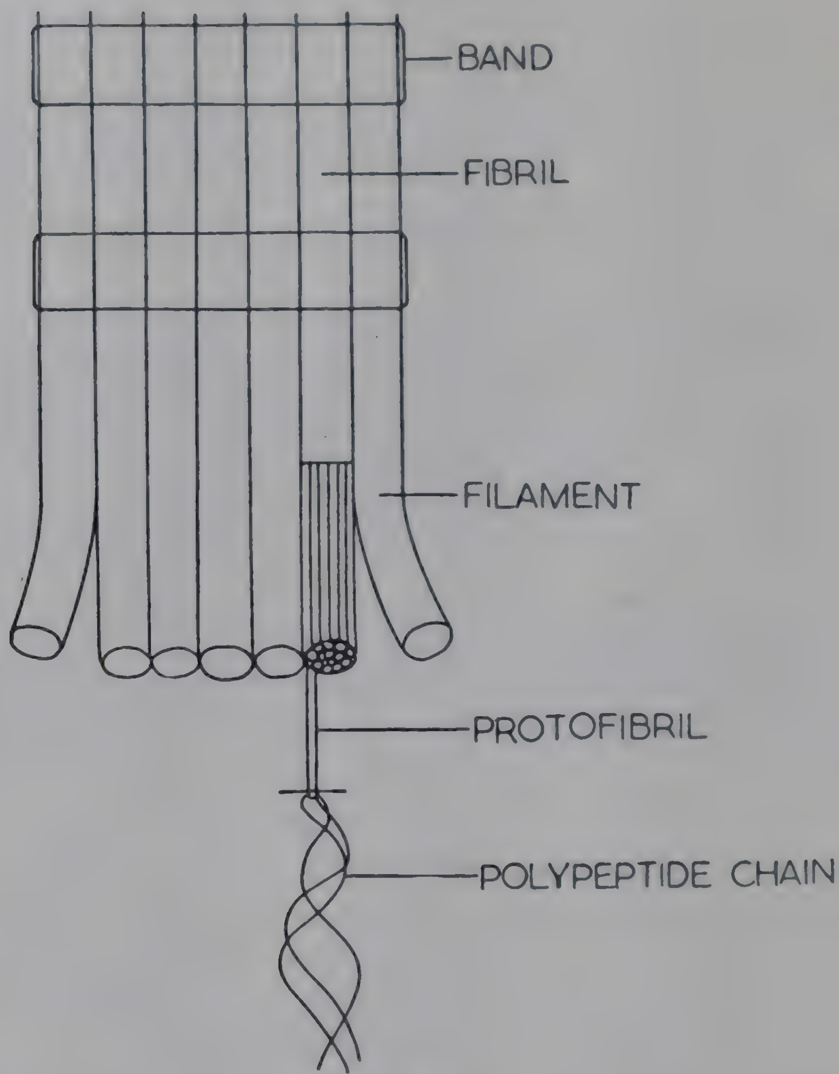


FIG. 1
Scheme showing various levels of structure within the collagen fibril.

1955), have suggested that the bands arise when certain regions of the protofibrils fold back upon themselves or when they overlap in special ways.

Various electron microscopists, however, have drawn attention to the peculiar helical forms which the collagen *fibril* may assume under a variety of experimental conditions (Wyckoff, 1949; Reed and Rudall, 1948; Swerdlow and Stromberg, 1955; Reed, Wood and Keech, 1956). The grinding of collagen fibrils in a Waring blendor, an operation frequently carried out in electron microscopic preparations, may lead to the formation of tactoidal structures, which appear to follow a helical course around the fibril axis (Keech, 1955; Swerdlow and Stromberg, 1955). Their formation seems to involve a loosening of the fibril along a helical path, so that the tactoids

appear as quite distinct structures (Fig. 2). Similar helical tactoids have been observed during the shrinkage of native collagen (Nutting and Borasky, 1948) and of tanned collagen fibrils (Burton, Reed and Wood, 1956) and again in fibrils exposed to reagents over almost the entire range of pH value (Reed, Wood and Keech, 1956). It is important to note that the direction of the helix determined by the helical tactoids has no preferred direction. Tactoids with both left- and right-hand spiral directions occur with approximately equal frequency, whilst sometimes both left- and right-hand spirals may be observed within the *same* fibril (Fig. 3). Tactoidal structures are often encountered during studies of the enzymatic digestion of collagen fibrils and also in the reconstitution of highly organized structures from solutions of material extracted by various chemical reagents. Keech (1955), for example, has shown that they arise when collagen is digested by trypsin or by collagenase, whilst Schmitt, Gross and Highberger (1955) have observed them when solutions of ichthyocol collagen in acetic acid are precipitated by the addition of glycoprotein. Whether such tactoids, however, were originally arranged in a helical manner within the fibril, still needs further investigation.

The helical tactoids can undergo subdivision into smaller structures (filaments), along lines which again follow a spiral course. As such subdivision occurs, the fibril appears to be composed of *two* sets of filaments, which cross each other in spiral fashion (Fig. 4). Such cross-helical arrays of filaments arise when the collagen fibril is treated with acid solutions (Wyckoff, 1950; Randall, 1953; Reed *et al.*, 1956). They have also been observed in the fibrils of the cuticle collagen which is secreted by the epidermal cells of the earthworm (Reed and Rudall, 1948). The presence of filaments arranged in cross-helical fashion within the fibril is also indicated by the fact already mentioned, namely that tactoids based on both left- and right-hand spirals may be observed within one and the same fibril. It is important to note also that the formation of helical tactoids and of cross-helical arrays of filaments appears to involve a loosening of certain filaments in the intact fibril *along a spiral line which crosses the bands at a definite angle.* How such a loosening along spiral paths can occur, if the filaments are arranged *parallel* to the fibril axis, as current models for the fibril architecture imply, is difficult to conceive. The fibril architecture must be such that:

(*a*) helical tactoids may be produced by the loosening of the fibril structure along a spiral path (around the fibril axis) and which cuts the bands at a definite angle;
(*b*) cross-helical arrays of filaments arise when the fibril is opened up;
(*c*) when helical tactoids and cross-helical arrays of filaments are produced, there is no great reduction in fibril or in filament length;

and these conditions are difficult to satisfy if the filaments are arranged in parallel fashion.

There are various ways of accounting for the helical structures manifested by the collagen fibril. Perhaps the most simple is as follows: Let us suppose that the *protofibrils*, the coiled-coil system of three polypeptide chains as indicated by the X-ray diffraction studies, are twisted together to form *filaments*. The *filaments* are then organized together in a steep, spiral fashion to form *two* independent identical *strands*. The two *strands* then spiral around each other to make up the complete fibril. Further, if in the normal intact fibril, the interspiralling of the two strands is steep, then all the filaments would lie very nearly parallel to the fibril axis, as indicated by the X-ray diffraction patterns (see Fig. 5).

Regarding the organization of the protofibrils into filaments, the electron microscope can provide little information, since the study of this level of structure demands a high degree of resolution which is beyond the scope of most present-day instruments. Since, however, the protofibrils appear to possess a coiled configuration, it is likely that they do in fact twist together in forming the filaments. The filaments on the other hand appear to be organized in very steep spirals when forming the strands.

It should be noted that certain authors have considered that the bands themselves follow a spiral path around the fibril axis (Swerdlow and Stromberg, 1955). Even if this disposition of the bands were substantiated, the spiral paths followed by the filaments appear to lie in directions quite distinct from that followed by the bands.

On the basis of the proposed model, the helical tactoids could arise by the loosening of *certain* filaments from their connections with their neighbours in the strands. The subdivision of the helical tactoids into thinner structures (and ultimately into filaments),

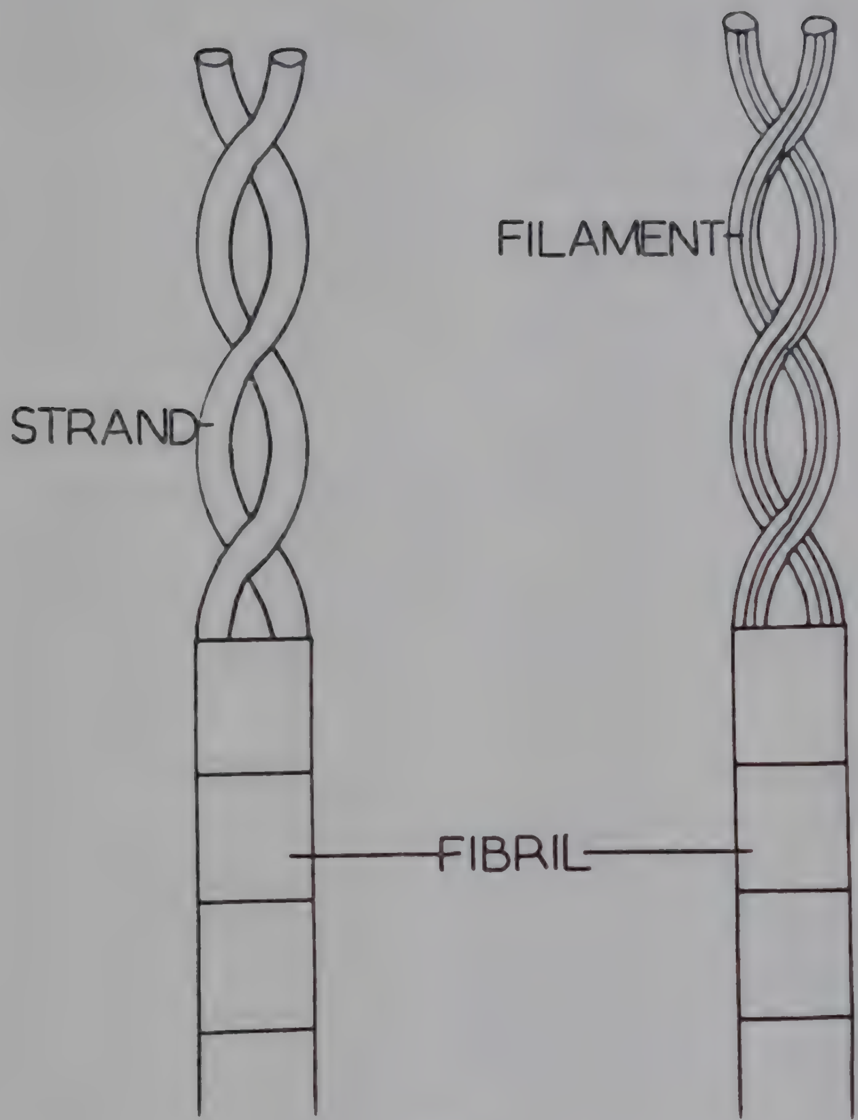


FIG. 5
The suggested scheme for the architecture of the collagen fibril, based on the spiralling of two strands.

suggests that the filaments are not all of the same chemical composition, that some are loosened more easily than others and that they are *regularly* arranged within the strands. If the filaments were of identical composition, it is difficult to explain the regularity in size of the helical tactoids and their regular subdivision into filaments. The presence of filaments of different chemical composition, regularly arranged in spiral fashion within the fibril, does not of course agree with the conception of a *single* type of protofibrillar unit. Whether better agreement with the X-ray diffraction data would come not from *one*, but from a *series* of protofibrillar units, each based on different numbers and types of polypeptide chains, remains

to be seen. Some workers in the X-ray diffraction field, however, have already stated that the assumption that a single structure can be built to account for the entire high-angle diffraction may well not be justified (Cowan *et al.*, 1955).

It is well known that fractions with differing physical and chemical properties may be extracted from the collagen fibril by treatment with various reagents. Furthermore, when the material extracted is 'reconstituted' by various means (salting out, pH change, temperature change, precipitation with additives, etc.), the electron microscope shows that a wide range of structural forms may be obtained, ranging from disordered gels to highly organized fibrous structures, closely similar to the original collagen fibrils. However, the correlation of the results obtained by these two types of approach, biochemical and electron-optical, has so far proved very difficult. How far, for example, the reconstituted forms represent building units in the original fibril is still undecided and until the biochemical procedures can be more closely coupled with electron microscope observations, is likely to remain so. It is possible that the new model, based on filaments of different chemical composition, may be useful in removing some of the difficulties in this connection. Firstly, there is the question of how far the fibril form is maintained under the conditions of extraction. Banga *et al.* (1956) have stated, for example, that all the procollagen may be extracted from collagen fibres with the retention of fibre structure. Burton, Hall *et al.* (1955) have shown that when skin collagen is extracted with alkaline buffer solutions, a procedure which leads to the release of protein fragments rich in hydroxyproline, the fibre form is not completely destroyed. Then there is the question of the form in which the material is extracted and its form in solution. Is the filament structure retained in solution or does the extraction involve breakdown of the filaments into their component protofibrils or even into separate polypeptide chains? It seems significant that whenever the reconstituted forms of the extract show highly organized structures, closely resembling the original banded fibrils, the electron micrographs almost invariably show many filaments in the background. On the other hand, if the filament structure is more or less destroyed and the protofibrils are unravelled to form polypeptide chains, during reconstitution it may not be possible to reform the coiled protofibril and hence the less organized forms of material may be accounted for.

It has been suggested that the helical structure under discussion might arise by purely physical means, e.g. differential shrinkage or swelling of the collagen fibril (Rudall, 1956). Thus it is known that a bundle of filaments arranged in parallel fashion could assume a spiral form, provided the outer filaments of the bundle shrink at a rate different from those in its core. Though spiral structures do indeed arise during the shrinkage of both native and of tanned collagen (see above), it seems likely that shrinkage involves a reduction in band spacing, i.e. the bands come closer together, as illustrated in Fig. 6. The formation of the spiral structures, however, appears possible *without* any appreciable change in band spacing and also at temperatures below those at which shrinkage might be expected to occur. Hence the balance of evidence at the moment favours the presence of filaments in cross-helical array, within the relatively unaltered fibril, as has been considered above.

GROUP DISCUSSION

Dr. Gross stated that he was surprised at the flatness of the tactoids and felt sure that strict adherence to a particular angle of spiralling could not be observed. He suggested that the effect could more likely be ascribed to variations in the alignment of the filaments brought about during the deposition of the material on the grid rather than the twisting of parallel filaments in the contraction process.

Dr. Reed felt that this might be an explanation of this phenomenon but assessed Dr. Gross's statement as merely a matter of opinion.

In support of his contention, Dr. Gross mentioned that this phenomenon was often only observable in small lengths of certain fibrils, and he felt it was a function of the method of preparation and could be obviated by gentle procedures. Dr. Reed agreed that preparation might play an important part in the production of the helical structures but that since they homogenized their preparations prior to treatment they often found considerable lengths affected.

Dr. Bear pointed out that collagen density is as high as the molar volumes of the constituents would permit, hence intrafibrillar filaments would have to be very close packed. A twisting of gross elements would make it difficult for observed densities to be attained. Also, thus far it has not been apparent that the small-angle X-ray diagram is compatible with gross filament twists. The wide-angle patterns do indicate finer coils at the molecular level. He suggested that Dr. Reed's observations may be

the result of a preferred manner of spreading of shrinkage along fibrils, possibly related, for example, to the disposition of molecules within the fibrils. Molecular ends are likely to be arranged in staggered, perhaps helical, fashion along the fibrils because the molecules have lengths which are about four times the normal macroperiod.

Dr. Reed gave three more pieces of evidence in favour of his observations: (1) tendon collagen gives even better spiral structures than skin collagen; (2) Dr. Grassmann showed a picture of a procollagen fibril in which there existed two strands which appeared to spiral around one another, and (3) Dr. Fitton Jackson also showed fibrils in cross-section which showed localization of filaments into definite strands within the fibril.

Dr. Fitton Jackson did not feel that her observations demonstrated any spiralling of fibrils around one another, and that the possible existence of sub-fibrils within each fibril from adult tendon neither supported or detracted from Dr. Reed's contention.

Dr. Reed also drew attention to the fact that degradation studies, especially with enzymes, produced structures which were tactoidal in nature, and Dr. Gross's tropocollagen aggregates in this form as well.

Dr. Gross felt that this was inconclusive since any rigid rod-like structure such as tobacco mosaic virus could aggregate in the form of tactoidal bundles.

In answer to a question by Dr. Fitton Jackson, Dr. Reed stated that they had no quantitative figures for the incidence of spiral or tactoidal structures in their material.

Dr. Fitton Jackson inquired how Dr. Reed envisaged the tactoidal structures appearing from the spirals. To which he replied that he had not suggested that this occurred, merely that the spiral represented the initial stage of the phenomenon and that the fibres showed lines of weakness along the spiral. Referring to the last illustration in Dr. Reed's paper, Dr. Fitton Jackson inquired how he proposed that the spiralling filaments could bring about the bands which were normally observed in collagen.

Dr. Reed suggested in reply that whereas Dr. Bear envisaged the bands as alternate areas of perfection and imperfection in the packing of parallel fibres, he would wish to consider the bands as being composed of the areas where the helically wound filaments either crossed or lay apart from one another. In answer to a question by Dr. van den Hooff who found it difficult to correlate the birefringence with such a structure, Dr. Reed pointed out that the effects concerned were only observed in treated fibres when the birefringence would anyway be impaired.

Dr. D. S. Jackson suggested that the production of tactoids in the collagenase experiments might be due to the fact that owing to the close packing of the fibrils the enzyme was only able to penetrate at these specific

points; to which Dr. Reed replied that this indeed had been the very point he wished to make.

Dr. Neuberger said he failed to understand how the strands could unravel without uncoiling down the whole length of the filament; to which Dr. Reed replied that he considered it was merely a loosening of the filaments within the strands so that they still remained intertwined.

Dr. Bear showed a series of small-angle X-ray diagrams obtained from kangaroo-tail tendon at a variety of moisture contents (Fig. 5 of Rougvie and Bear, 1953, *loc. cit.*, p. 321). The most moist samples yield diagrams whose reflections are of equal size, as is expected of diffraction by smooth, parallel-sided cylinders (fibrils). The 'fanned diagrams' of dry material have been given reasonably satisfactory interpretation in terms of variations in order and distortion occurring periodically along the fibrils. The fanning does, however, show some resemblance to the diffraction expected of gross helical twists, but the X-ray diagram on this basis would indicate a coil pitch equal to one macroperiod; the twists of Dr. Reed's helices extend over a number of macroperiods. If, in spite of this difficulty, there should be a relationship between Dr. Reed's phenomenon and the diffraction fanning, it would be interesting to compare the pitch angles of Dr. Reed's fibrils with the X-ray 'fanning angle', which departs about 24° from the fibrillar axis or rises at about 66° to the equatorial (cross-sectional) plane.

Dr. Reed replied that the steepest angle of the spiral was about 70°. He drew Dr. Bear's attention to the fact that, although he had suggested that the phenomenon might be due to differential shrinkage, it does in fact occur at temperatures so low that one could not expect shrinkage to occur. But even if one accepts this explanation, it does in fact strengthen the point which he was wishing to make, namely that certain specific regions in the fibril were more sensitive to shrinkage than others.

AMINO-ACID SEQUENCES OF COLLAGEN

W. GRASSMANN, K. HANNIG, H. ENDRES AND A. RIEDEL

The highly differentiated fine structure of collagen (Hall, 1942; Wolpers, 1943, 1944; Schmitt, 1948; Grassmann, 1952; Hofmann, 1952; Nemetschek, 1955) and procollagen (Kühn *et al.*, 1956) visible in the electron microscope indicates that there is a relation between the molecular structure of these fibrous proteins and their electron microscopic picture. In order to gain an insight into the amino-acid sequence of collagen and pro-collagen, we have begun to separate the degradation peptides, obtained by digestion with crystalline trypsin, by means of electrophoretic and chromatographic methods and to study their chemical constitution.

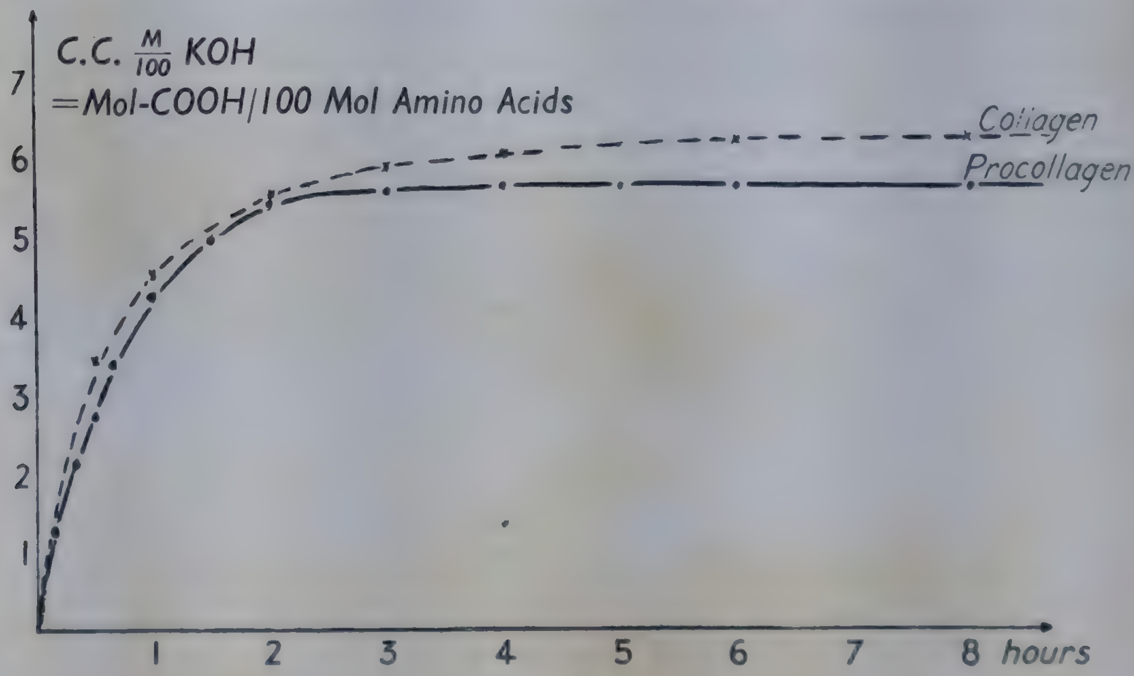


FIG. 1
Tryptic digestion of
X - - - X Collagen and
·——· Procollagen.

The tryptic digestion of collagen and procollagen comes to a distinct endpoint after the digestion of a part of the peptide linkages. Thus degradation products with an average chain length of about 18 amino acids are formed (Fig. 1). The resulting peptide mixture

Fig. 2
Human dermal collagen fibrils after fragmentation in a Waring blender. Arrows show the helical tactoids.

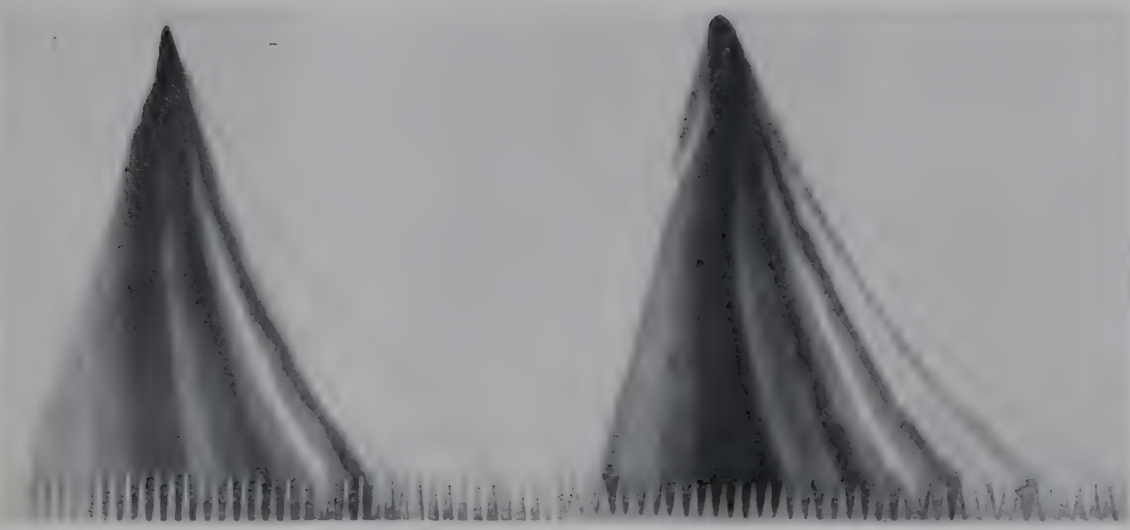


FIG. 2
Electrophoretic separation patterns after tryptic degradation at pH 4.9 of
(*a*) collagen and
(*b*) procollagen.

FIG. 4
Further separation of the neutral fraction by electrophoresis at pH 2.3.

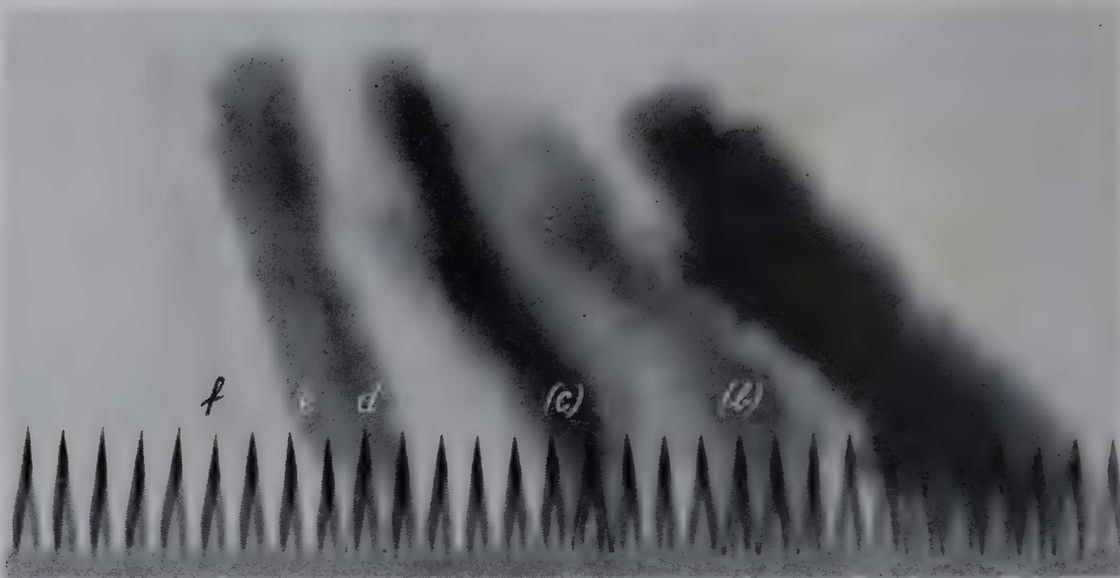
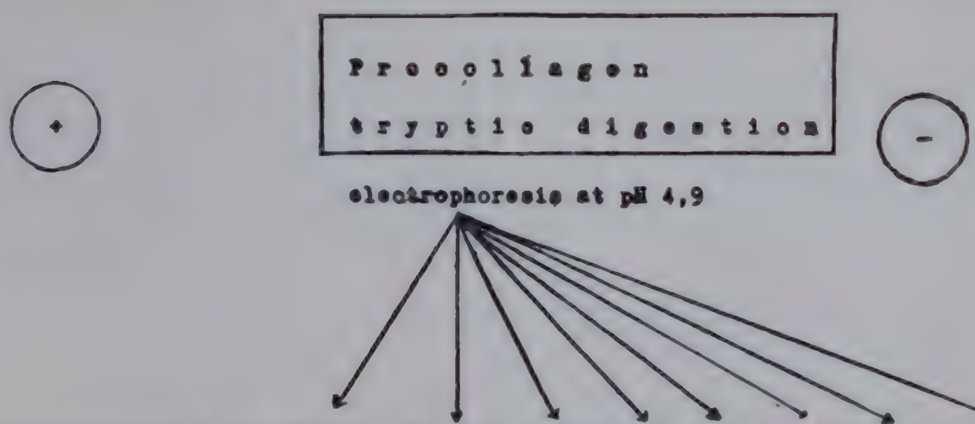


FIG. 5
Further separation of the basic fraction B_4 in 4 subfractions at pH 2.3.

has a complicated composition. The separation of such complicated mixtures of peptides having such great chain lengths is by no means easy. Among other things, loose addition compounds of such polypeptides are apparently sometimes formed whose complete separation cannot be accomplished in one single operation. We have found the continuous electrophoresis (Grassmann and Hannig, 1953) to be a suitable means for this purpose. The separation patterns obtained by this method from both collagen and procollagen degradation products at a pH value of 4.9 (Fig. 2 correspond fairly exactly (Grassmann, Endres and Steber, 1954). This and the quantitative amino-acid analysis indicate that there is a similar correspondence in the case of the amino acid sequences. As to procollagen, it can be purified by repeated recrystallization. The separation pattern is sharper which indicates that this material is of a more homogeneous nature. We have therefore mainly used such recrystallized procollagen in our experiments. Quantitative checks were made at all stages of the separations. Fig. 3 shows a schematic



Fraction	S	N	B_6	B_5	B_4	B_3	B_2	B_1
weight %	19.23	57.37	7.21	12.4	3.2	0.2	0.25	0.15
% N of total N	14.1	60.6	6.1	13.7	4.1	0.35	0.50	0.35

FIG. 3

Schematic diagram showing the electrophoretic isolation process of procollagen-peptides resulting from tryptic cleavage.
S = Acid fraction, N = neutral fraction, B = basic fractions.

diagram of the separation process and the yields obtained thereby.

Only very few of the fractions resulting from a single separation process at pH 4.9 are homogeneous. Most of them can be further

X

TABLE I

THE AMINO-ACID COMPOSITION OF THE HOMOGENEOUS PEPTIDES OBTAINED BY ELECTROPHORETIC SEPARATION OF THE TRYPTIC HYDROLYSATES

		polar							non-polar			
Fraction		B_1	$B_{6}a$	S_a	B_3	$B_{5}b$	S_b	S_d	S_e	B_{4c}	$B_{5}d$ II	S_f
Yield (effect)	weight (per cent)	0,15	0,03	0,20	0,20	0,53	0,3	0,75	2,6	0,52	0,8	1,6
Total amount approx.	weight (per cent)	0,15	0,07	0,35	0,2	1,20	0,75	1,65	4,7	1,3	3,5	3,15
	per cent N	0,3	0,1	0,33	0,35	1,3	0,77	1,65	4,7	1,4	3,4	3,05
Total N (per cent)		29,5	22,1	14,2	26,2	16,0	15,4	15,3	15,2	15,9	14,5	14,3
Gly		1	1	5	5	6	14	12	24	10	6	14
Ala		—	—	—	—	3	5	5	12	5	3	3
Val		—	—	—	—	1	1	1	1	1	1	—
Leu+ILeu		—	—	—	—	2	1	1	2	4	1	1
Phe		—	—	—	—	—	—	—	1	—	—	1
Ser		—	—	—	—	—	1	1	3	—	1	2-3
Thr		—	—	1	—	—	1	—	—	2	—	1
Tyr		—	—	—	—	—	—	—	—	—	—	2
Met		—	—	—	—	—	—	—	1	—	—	—

TABLE I, *continued*

	polar								*non-polar*		
Fraction	B_1	$B_{6}a$	S_a	B_3	$B_{5}b$	S_b	S_d	S_e	$B_{4}c$	$B_{5}d$ II	S_f
Pro	—	—	—	—	1	5	6	11	3	2	10
Hypro	—	—	—	—	1	4	4	7	3	3	5
Asp	—	—	2	—	1	3	3	4	—	—	2
Glu	—	1	3	—	1	5	4	8	—	1	2
Lys	—	—	—	1	1	2	1	2	—	1	—
Hylys	—	—	1	—	1	—	—	—	2	—	—
His	1	—	—	—	1	—	—	—	2	—	—
Arg	1	1	1	4	1	2	1-2	3	1	1	—
Amide-N	—	—	—	—		(1)	(2)	(4)	—	—	—
Chain length	3	3	13	10	20	44	39-40	79	33	20	43-44
Glycine (mol. per cent)	33,3	33,3	38,5	50,0	30,0	31,8	30,8	29,2	30,3	30,0	32,6
Pro+Hypro (mol. per cent)	—	—	—	—	10,0	20,5	25,5	22,8	18,2	25,0	34,8
Diamino acids (mol. per cent)	66,6	33,3	15,4	50,0	20,0	9,1	5-8	6,4	15,2	10,0	—
Dicarboxylic acids (mol. per cent)	—	33,3	38,5	—	10,0	18,2	18,0	15,2	—	5,0	9,3
Sum of diamino and dicarboxylic amino-acids	66,6	66,6	53,9	50,0	30,0	27,3	23-26	21,6	15,2	15,0	9,3

separated into sub-fractions, either by electrophoresis at another pH value or by means of chromatography. Fig. 4, for example, shows the further separation of the neutral fraction by electrophoresis at pH 2.3. Fig. 5 shows the separation of the basic fraction B_4 into four sub-fractions, also at a pH value of 2.3. We do not consider a fraction homogeneous until we have found that no further separation by electrophoresis is possible at three pH values (2.3; 4.9; 9.2). Moreover, this criterion must apply in four different solvent mixtures by chromatography. Finally, the quantitative amino-acid analysis (Grassmann, Hannig and Plöckl, 1955) must result in a clear-cut and integral ratio of all amino acids. These strict requirements which hitherto have not been applied on ascertaining the constitution of proteins and protein degradation products appear to be necessary in our opinion. At present we have obtained about eleven peptides that fulfil such requirements.

Table I contains the actual yield of the individual peptides in per cent by weight and an estimate on the quantitative percentage in which they are contained in the degradation mixture. Since the purification process is at all stages checked by quantitative analyses, this estimate is fairly exact.

The accuracy of the analyses from which we obtain the stoichiometrical ratio of the amino acids may be illustrated by two examples, fraction S_a and fraction B_{4c} (Table II).

Table III illustrates the determination of the constitution of Fraction B_1 which is homogeneous after the first separation. However, it is obtained in insignificant quantities only. The sequence is gly-arg-his (Grassmann, unpublished).

Glycine is present in fairly uniform quantities in all fractions as shown in Table I. It usually amounts to one-third of the total amino acids, and only in two cases – the quantitatively rather small fraction S_a and B_3 – did we find values of 38 and 50 mol per cent of the total amino acids respectively.

If the peptides so far obtained are listed in the order of their molecular contents of highly polar amino acids, i.e. of the sum of diamino and dicarboxylic acids, on the one hand, and in the order of their contents of the amino acids proline and hydroxyproline, on the other hand, we find that if the order of the one group of amino acids increases, in the other group it decreases. Split peptides with a high content of polar amino acids contain little or no proline and hydroxyproline and vice versa. These findings alone render the

TABLE II

STOICHIOMETRICAL RATIO OF THE AMINO ACIDS OF FRACTIONS S_a AND B_{4c}

	S_a		B_{4c}	
	Total N: 14,3 per cent *Amide N: 0,05 per cent*		*Total N: 14,3 per cent* *Amide N: 0,00 per cent*	
	Mole ratio found	*Mole ratio rounded*	*Mole ratio found*	*Mole ratio rounded*
Gly	4,95	5	10,3	10
Ala	—	—	5,1	5
Val	—	—	1,15	1
Leu+Ileu	—	—	3,8	4
Phe	—	—	—	—
Ser	—	—	—	—
Thr	0,97	1	1,96	2
Tyr	—	—	—	—
Meth	—	—	—	—
Pro	—	—	2,95	3
Hypro	—	—	3,40	3
Asp	2,04	2	—	—
Glu	3,03	3	—	—
Lys	—	—	—	—
Hylys	1,00	1	1,67	2
His	—	—	1,97	2
Arg	1,00	1	1,2	1
	12,99	13	33,5	33

TABLE III

DETERMINATION OF THE CONSTITUTION OF FRACTION B_1

FRACTION B1 (GLY-ARG-HIS)

	*Per cent of total N**	*Mole per cent*
Glycine	12,7	33,6
Histidine	37,8	33,3
Arginine	49,7	33,1
	100,2	100,0

N-terminal amino acid†: Glycine
C-terminal amino acid‡: Histidine

* W. Grassmann, K. Hannig and M. Plöckl, *Hoppe-Seyl. Z. physiol. Chem.*, **305**, 21 (1956).
† F. Sanger, *Biochem. J.*, **39**, 507 (1945).
‡ W. Grassmann, H. Hörmann and H. Endres, *Ber.*, **86**, 1477 (1953).

theory of Bergmann and Niemann (1936) improbable. They assumed a regular recurrence of tripeptide units consisting of 1 glycine, 1 proline or hydroxyproline and one other amino acid. In conformity with other authors (Schroeder *et al.*, 1953, 1954; Kroner *et al.*, 1953, 1955; Bear, 1952) the results rather indicate an alternation of non-polar areas that are rich in proline and hydroxyproline, and of polar ranges poor in amino acids which might account for the dark and light bands (Schroeder *et al.*, 1954).

In order to gain further insight on the proline and hydroxyproline containing sequences, we have studied initially the fraction S_f which has the highest content of amino acids of all fractions. At a pH value of 4.9 it migrates slowly towards the anode, and at pH 2.3 its migration path shows the least deviation of all acid peptides towards the cathode. This, of course, facilitates its isolation. The actually isolated quantity of this fraction is equivalent to 1.55 per cent of the total nitrogen, and 1.6 per cent of the weight of procollagen. Its actual proportion in the mixture is about 3 per cent. The fraction makes up almost 5 per cent of the total content of proline and hydroxyproline.

TABLE IV

QUANTITATIVE AMINO-ACID ANALYSIS OF THE FRACTION S_f

Total N: 14,3 per cent
Amide N: 0,05 per cent

	Per cent of total N		*Mole ratio found*		*Mole ratio rounded*
	I	II	I	II	
Gly	31,40	31,90	14,00	13,95	14
Ala	6,53	6,18	2,92	2,70	3
Leu+Ileu	1,68	1,95	0,75	0,85	1
Phe	2,44	2,63	1,09	1,15	1
Ser	6,05	5,15	2,70	2,25	2-3
Thr	1,88	1,83	0,84	0,80	1
Tyr	4,10	2,97	1,83	1,30	2
Pro	22,9	23,40	10,2	10,2	10
Hypro	11,42	11,35	5,2	4,95	5
Asp	4,70	4,74	2,05	2,07	2
Glu	4,36	4,70	1,95	2,05	2
	97,46	96,80	43,53	42,27	43-44

I = Hydrolysis with 6 n HCl, 24 hours at 100° C.
II = Hydrolysis with 6 n HCl, 48 hours at 100° C.

The amino-acid analysis (Table IV) shows the complete absence of basic amino acids and of amide nitrogen. The chain length established by the amino-acid analysis is 43 to 44 amino acids, which is equivalent to a molecular weight of 3900 to 4000. Glycine, and only glycine, is found in the N-terminal residue by the Sanger method and only aspartic acid in the C-terminal residue by the reduction method and by the use of carboxypeptidase (Grassmann, Hörmann and Endres, 1953, 1954, 1955). The yield of aspartic acid established by the reduction method was found to be equivalent to 0.7 mol for 43 amino acids.

The photometrical determination in the case of the DNP-derivative showed a molecular weight of 3717 for the free peptide. The potentiometric titration of the DNP-derivative shows a carboxyl equivalent of 727. This corresponds to a molecular weight of 3635 referring to 5 carboxyl groups, namely one terminal carboxyl group and 4 carboxyl groups of the amino-dicarboxylic acids. The conformity of these values with the molecular weight derived from the amino-acid composition indicates chemical homogeneity, in addition to the chromatographic and electrophoretic examination.

Almost exactly one-third, that is to say 14, of the total amino acids are glycine, another one-third or 15 residues are proline and hydroxyproline. The remaining 15 to 16 residues are other amino acids.

Thus, as far as its composition is concerned, the fraction might very well be in line with the conception of Bergmann and Niemann. However, the examination of the amino-acid sequence led to results that entirely contradict any such assumption.

Carboxypeptidase quite readily splits off 2 mol of aspartic acid (Fig. 6). Leucine, glutamic acid, and phenyl-alanine follow each at a decreasing rate. Neither proline nor hydroxyproline are split off, however. It has been shown that C-terminal leucine is much more rapidly split off by carboxypeptidase than aspartic acid (Neurath and Schwert, 1950). The sequence in the C-terminal must therefore be leu-asp-asp-COOH. If the sequence were asp-leu-asp-COOH, leucine would have to be split off much more quickly than the second mol of the aspartic acid. An analogous consideration holds true in the case of phenyl-alanine and glutamic acid. Hence it is highly probable that the sequence in the C-terminal residue is

. . . . phe-glu-leu-asp-asp-COOH.

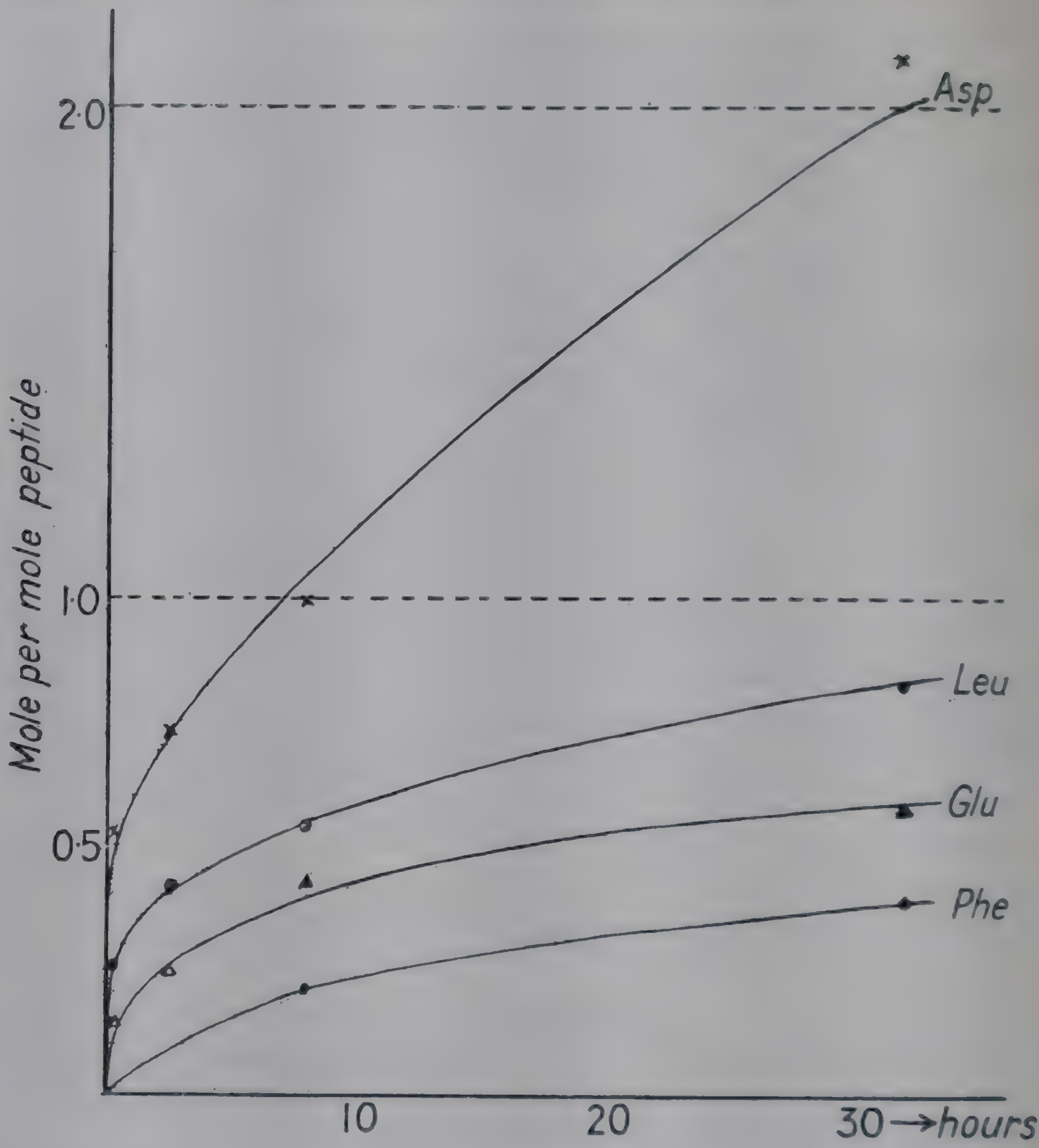


Fig. 6

Quantitative determination of the amino acids split off from the procollagen fraction S_f by carboxypeptidase after different times.

At any rate, it can be said with certainty that proline and hydroxyproline are not among the first five amino acids in the C-terminal residue.

Hydrolysis with aminopolypeptidase from yeast (Grassmann, 1934) shows that, besides glycine, also serine, threonine and alanine are constituents of the N-terminal residue (Fig. 7). The fact that also a small quantity of leucine (0.2 mol) is split off by aminopeptidase is somewhat strange, since this amino acid is present only once, according to the amino-acid analysis, and since it is also found in the C-terminal residue.

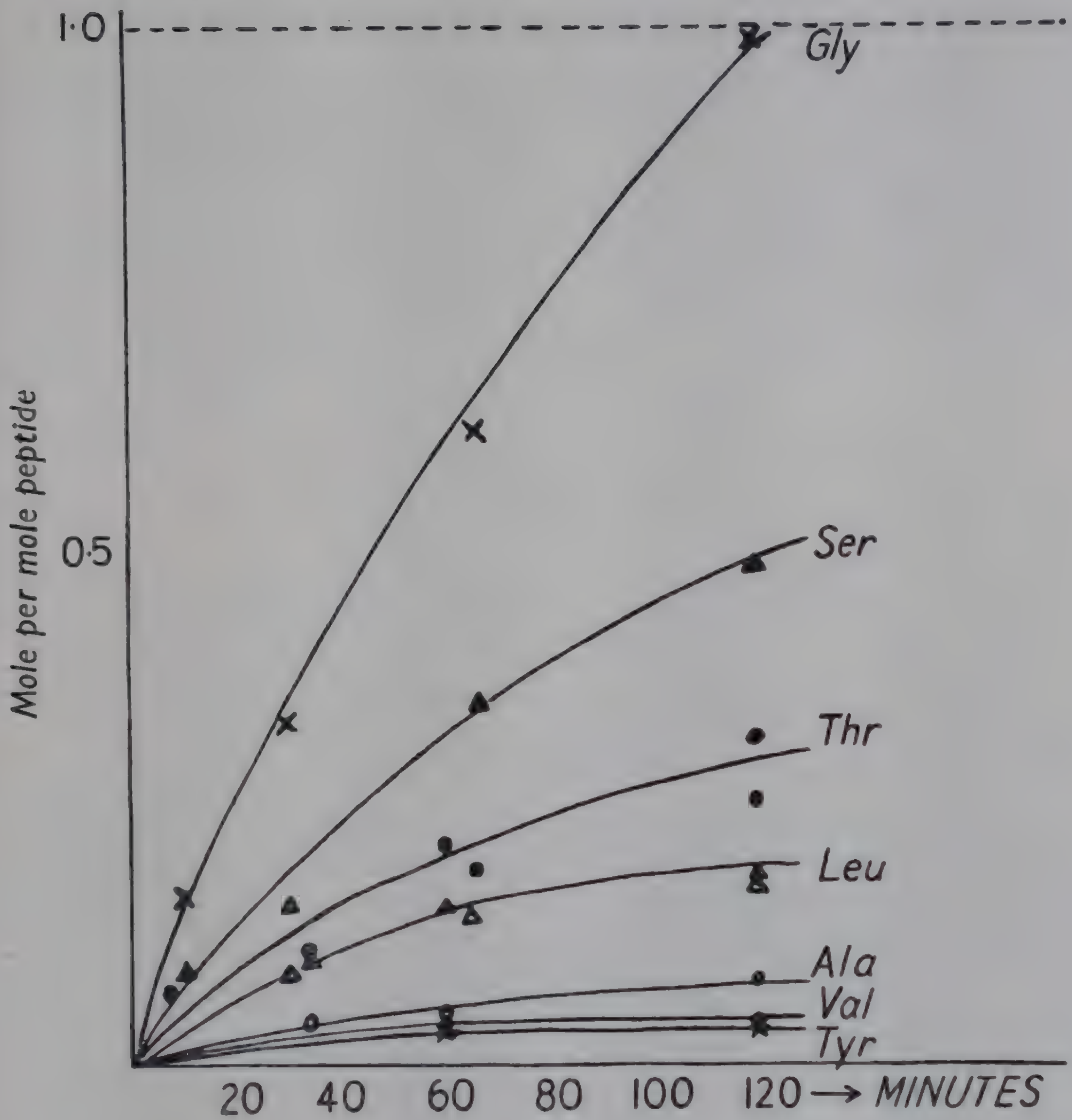


FIG. 7
Quantitative determination of the amino acids split off from the procollagen fraction S_F by aminopeptidase after different times.

The partial hydrolysis of the DNP-derivative resulted in DNP-peptides which, apart from terminal glycine, contained serine, threonine, alanine, further glycine, and again small quantities of leucine. Proline and hydroxyproline were found neither among the amino acids split off by aminopeptidase nor among the DNP-peptides resulting from the partial hydrolysis of the DNP-derivative. Our experiments have not been sufficient to make possible a final determination of the amino-acid sequence in the N-terminal residue. It is certain, however, that glycine, serine, alanine, threonine and one or two more glycine residues must be among the first six amino

acids from the N-terminal residue, whereas proline and hydroxyproline cannot be among the first six.

The amino acids from the middle of the peptide are found among the peptides obtained as DNP-free peptides in the partial hydrolysis of the DNP-derivative. They contain considerable quantities of proline, hydroxyproline and glycine, and also serine, alanine and tyrosine. The constitution of the peptide can thus be set down as follows:

FORMULA I

CONSTITUTION OF THE PEPTIDE Sf

$$\underbrace{\text{H}_2\text{N-gly-(ser, thr, leu, ala, gly)-}}_{\text{N-terminal section}}$$

$$\underbrace{\text{[10 pro, 5 hypro, 12 gly, 2 ala, 1-2 ser, 2 tyr, 1 glu]}}_{\text{centre section}}$$

$$\underbrace{\text{-phe-glu-leu-asp-asp-COOH}}_{\text{C-terminal section}}$$

If it is assumed that glutamic acid and tyrosine are actually contained in the N-terminal or C-terminal section – our experiments do not exclude this possibility – it follows that the middle part must be made up of 10 prolines and 5 hydroxyprolines on the one hand, and about the same number of very simple amino acids, namely 12 glycine, 2 alanine and 1-2 serine, on the other.

W. A. Schroeder *et al.* (1953, 1954) and Kroner *et al.* (1953, 1955) earlier, found gly-pro, hypro-gly and gly-(pro, hypro)-gly as disintegration products by means of partial hydrolysis with acids. These authors are of the opinion that the tetra-peptide units gly-pro-hypro-gly are essential structural elements of the crystalline parts of the fibril. The findings of Gustavson (1953), who showed that the thermo-stability of collagenous fibrils increases with their proline and hydroxyproline contents, are also on the same line.

Our findings support the conception of Schroeder and apparently prove the existence of a non-polar part containing about 30 amino acids whose sequence is very similar to the one assumed by Schroeder.

We should like to postpone a more thorough discussion of our findings relating to the structure models recently suggested by Ramachandran and Kartha (1955), Rich and Crick (1955) and

Cowan *et al.* (1955) until after we have fully explored the amino-acid sequence of the middle part. At any rate, its length of about 30 residues would correspond to one turn in the last model proposed by Ramachandran and it would be of a magnitude that could correspond to a single light band.

For any further determination of the collagen structure it will be of particular significance whether the *entire* proline and hydroxy-proline is present in aggregated groups of the type shown and whether it will be possible to establish a relation of this sequence with the electron microscope picture.

GROUP DISCUSSION

Dr. Bear remarked that, in the most recently proposed 3-chain coiled-coil models for the polypeptide chain configuration in collagen, the typical asymmetric units of the structure are 3-residue sequences rather than 4-residue ones. The models are most easily built upon repeated gly-pro-hypro sequences, or variations therefrom involving alterations of the last two of the three residues.

Dr. Grassmann agreed and pointed out that in the sequence G-R_2-R_3, postulated in the most recent model (Ramachandran *et al.*) G *must* be glycine or a 'glycine-like' amino acid, whereas R_2 and R_3 *can* be amino acids of the proline type. Although he considered any discussion of the sequence in the central 30-unit sequence to be premature, he pointed out that even a sequence glycine-glycine-proline would not be in contradiction with the model. Five peptides of the sequence glycine-proline-hydroxy-proline and five other ones of the sequence glycine-glycine-proline (where glycine represents a 'glycine-like' residue) would be in satisfying agreement with his analytical results.

Dr. Bear observed that at the C-terminal end of the S_f peptide glycine did not occur in every third position.

Dr. Grassmann said that this was really a surprising result. His other results show rather good agreement with the assumption of a regular periodicity of glycine-residues.

Dr. Bear said that 'anomalous' sequences of this sort will be important in determining whether different structures will need to be postulated, say between the portions located at bands and interbands of the fibril, or between the main length of the molecule and dangling appendages. He then showed a photograph of the 3-chain coiled-coil type of structure now favoured from physical evidence for the major portion of the collagen molecule.

In reply to Dr. Orekhovitch, Dr. Grassmann summarized the procedure for determining the C-terminal sequence with carboxypeptidase. Dr. Orekhovitch said he was sceptical about the method and suggested that the enzyme might be contaminated by a proteinase. Dr. Grassmann said his enzyme had been recrystallized five times and that any chymotrypsin could be inhibited by di-isopropyl fluorophosphate. Dr. Orekhovitch said that even after ten recrystallizations carboxypeptidase still contained proteinase which could not be inhibited by di-isopropyl fluorophosphate and that he preferred chemical methods. Dr. Grassmann replied that a reduction method for determining the C-terminal residue gave results in quantitative agreement with the enzymic method but pointed out that there was no reliable chemical method for determining *stepwise* the C-terminal *sequence*.

Dr. Snellman suggested that if the trypsin used was pure then the fact that the S_f peptide contained no lysine or arginine means that S_f is a terminal peptide. Dr. Grassmann said this was possible and added that other peptides isolated did contain C-terminal lysine and arginine. Some peptides have been isolated where this is not true.

In reply to Dr. Neuberger, Dr. Grassmann said that he had used recrystallized trypsin and that several samples gave the same results as regards the end point of the reaction. Traces of other enzymes might be present but he did not think this affected his main conclusions. In reply to Dr. Consden, Dr. Grassmann said that trypsin was used because its reaction with the substrate had a well-defined end point. The collagen was heated to its shrinkage temperature before being treated with trypsin.

THE COMPOSITION OF BANDS AND INTERBANDS OF COLLAGEN FIBRILS[1]

RICHARD S. BEAR AND RICHARD S. MORGAN

Investigators of connective tissue most often encounter the collagenous component as macroscopically or microscopically visible *fibres* of diameters 2 to 200 μ. These are formed of sub-microscopic *fibrils* whose diameters may range from a few hundred to several thousand Ångström units. In some circumstances it is convenient to consider also *sub*fibrillar units, *filaments* and *protofibrils* (Schmitt, Hall and Jakus, 1942; Bear, 1952), which result from successively finer stages of fibrillar subdivision.

Experimental demonstration of the existence of filaments as significant natural fibrillar units in collagen is still uncertain, because some degree of accidental longitudinal cleavage within fibrils is to be expected during manipulative procedures. Protofibrils were originally 'the unit columnar arrays which, when associated laterally, form the collagen fibril', but as knowledge has progressed regarding collagen molecules the necessity for this term has diminished. The molecules, further described below, are the true thinnest elements involved in fibrillar formation.

The fibril, however, provides the unit of more direct interest in biological and medical problems. As a result of recent work the molecule is now well defined in several important respects, but fibrillar structure is much less well determined. This paper is devoted to the latter problem.

There are two chief aspects to fibrillar structure: (1) the manner in which the molecules unite transversely to their long axes in fibrillar cross-sections; and (2) the way in which their parallel long axes are shifted relative to each other along the fibrillar axis. Leaving the transverse fibrillar organization to await further experimental development, in this paper we turn chief attention to the second aspect, related to longitudinal disposition of molecules.

[1] This work was supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and also in part by Research Grant A-901 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service.

One of us (Bear, 1952) has already reviewed preliminary ideas regarding the structural meaning of the bands and interbands of the fibrillar macroperiod, and here we shall revise and extend these proposals in the light of new evidence. The development below is concerned chiefly with the composition of bands and interbands as these have relation to distribution of material along molecules and of molecules along the fibril.

EXPERIMENTAL ESTIMATION OF MATERIAL DISTRIBUTION ALONG FIBRIL AXES

Nemetschek, Grassmann and Hofmann (1955) and Burge and Randall (1955) have provided the principal studies of variations of material content along collagen fibrils as observed directly by densitometry of electronmicrographs. A disappointing difficulty of this kind of work, emphasized by the latter authors, is the variability of result obtained from one macroperiod or fibril to another. One may question whether this great degree of variability still obtains in specimens carefully prepared for small-angle X-ray diffraction (see Bear, 1952), because of fair reproducibility of patterns and the sharpness and number of layer lines obtained. It would, therefore, be of interest to learn what small-angle intensity data can indicate about the distribution of matter along collagen fibrils.

The most direct procedure would be to determine what crystallographers call a Patterson projection, expressing the frequency with which vectors of different size connect parcels of electron density along the fibrillar axis. These were made early in this laboratory for a favourable case believed to have phosphotungstate staining principally in the *a* band (the 'cross-over' sample of Bear, Bolduan and Salo, 1951). In this case vectors much like those expected between *a* and all other known bands, as judged from electron-optical measurements of band positions (Schmitt and Gross, 1948) seemed to dominate the Patterson plot. The result was not convincing, however, and was not published.

The chief difficulty with Patterson plots is that they lack resolution sufficient to separate interband vectors, probably because the original fibrillar structure has too many, insufficiently sharp bands to permit clear separation of interband vectors when plotted in the Patterson way. The chief fact that has been learned from Patterson plots is that moist collagen diffracts approximately as would a single

extensive band, covering 0.46 to 0.47 of the macroperiod (Kaesberg and Shurman, 1953; Tomlin and Worthington, 1956). This could equally well mean that the complementary fraction, 0.53 to 0.54, is applicable, since diffraction cannot ordinarily distinguish 'positive' from 'negative' (in the photographic sense). Both investigations agree that dry collagen is less simply treated.

The Patterson plots are feasible because observed intensities of X-ray diffraction orders can be used alone. To obtain direct distributions of electrons (Fourier plots) one requires phases, as well as amplitudes (square roots of intensities), for the several orders. The phases are not experimentally determinable from the X-ray data alone. One can approach this problem in an indirect way by using models, derived from examination of electron micrographs or assumed from inspection of the small-angle diffraction data, to calculate phases, which can then be used with X-ray amplitudes to make the direct plots. This has been done by Tomlin and Worthington (1956), who, however, made the assumption, contradicted by electron optical information, that there are cross-sectional planes of symmetry in the fibril.

Recently, to facilitate this type of indirect approach, we have been using an optical diffraction apparatus designed by Dr. H. W. Wyckoff, to whom we are also indebted for suggestions of ingenious special procedures along the way. In brief, we first design models based on electron-optical information and using features known to result in diffraction similar to that observed on X-ray patterns; drawings of these are made and photographically reduced; the resulting 'mask' is made to diffract monochromatic visible light (the green 'line' from a mercury arc); and the final optical diffraction pattern is analysed or compared directly with the appropriate X-ray diffraction pattern. Satisfactory models are then used for calculation of phases. The X-ray intensities employed are unpublished ones determined at various times for kangaroo tail tendon in this laboratory by Drs. O. E. A. Bolduan and T. C. Furnas, Jr., in essential agreement with data given by Tomlin and Worthington (1956). Actually, the models themselves are satisfactory for most present purposes, and although quantitative calculations have been applied for comparison of intensities predicted by the models with observed ones, we rely here chiefly on photographic presentation of the results.

ELECTRON DISTRIBUTION ALONG MOIST AND DRY FIBRILS

Initially we determined arbitrarily to limit the resolution of our models to spacings (40 to 45 Å.) corresponding to the fifteenth diffraction order and approximating the electron-optical resolution of bands. Less than 1 per cent of diffracted energy in the small-angle meridional system occurs beyond the fifteenth order, and any attempts to draw significant conclusions about smaller detail (except at wide angles) would be fruitless at present. The models were then constructed as combinations of rectangular apertures, each with the dimension along the fibril axis equal to or greater than the resolution.

We first sought a simple way of representing band structure, based largely on data published by Schmitt and Gross (1948) and by Nemetschek, Grassmann and Hofmann (1955). Inspection of this information suggested that the following bands, at the fractional locations along the macroperiod given, would be suitable: d, 0.00; e, 0.16; a_1, 0.30; a_2, 0.39; b_1, 0.54; b_2, 0.64; c, 0.84. These were selected as the most consistently observed, strongest bands, which for simplicity could be given equal weight to represent the major features of band structure. In the models these were given length along the fibril axis equal to the resolution (0.067 of the macroperiod). It follows also, from diffraction theory, that separate apertures of this size, hence all of them, cannot contribute much diffracted radiation to the neighbourhood of the fifteenth order, as is required by the X-ray diffraction data.

Fig. 1 shows a series of macroperiods containing only bands, along with the corresponding optical diffraction pattern. It is immediately reassuring that the intensities of the orders beyond the fifth resemble the corresponding area of the X-ray pattern for dry collagen (see Fig. 3), indicating that the chosen band model can appropriately be used to account for most of this part of the diffraction field. Important X-ray diffraction at the lower orders is, however, not accounted for by the simple model.

Inspection of the problem of how to introduce this low-order scatter into the model shows that this can be done very simply as follows: a new, longer 'background' aperture must be introduced, which in particular should not add intensity near the weak fifth and tenth diffraction orders. Diffraction theory readily shows that this can be accomplished if the length of the background covers a

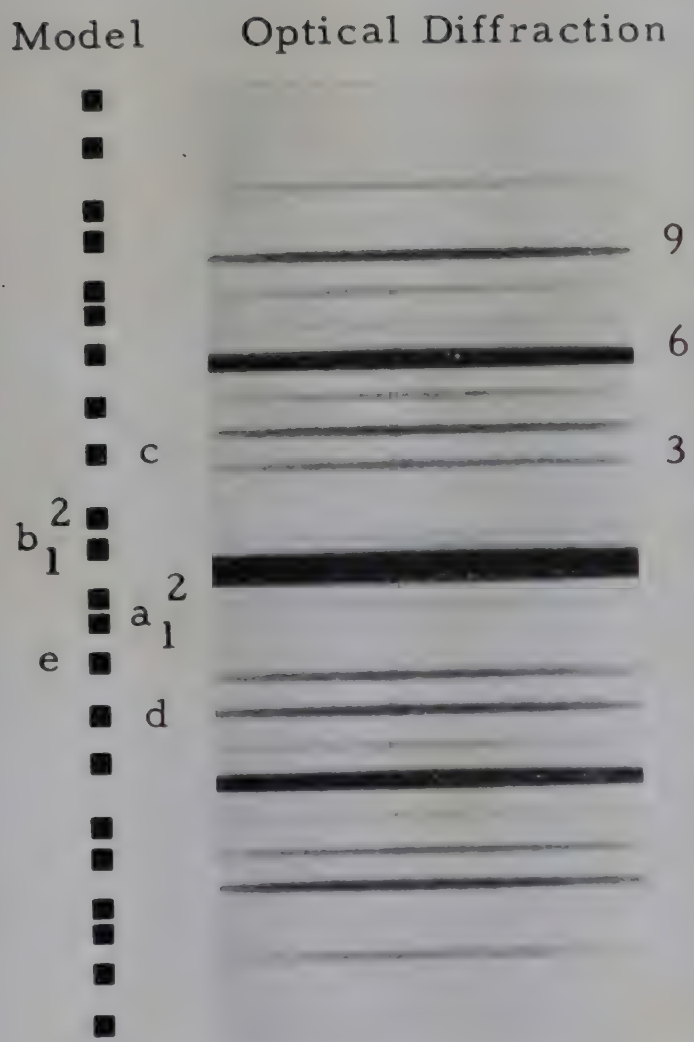


FIG. 1

Diffraction by a simple pattern of bands. Rectangular blocks, representing bands d, e, a_1, a_2, b_1, b_2 and c, are repeated vertically a number of times as shown on the left. A much reduced photographic negative of the model yields the optical diffraction reproduced on the right. Layer-line indices of the diffraction are indicated.

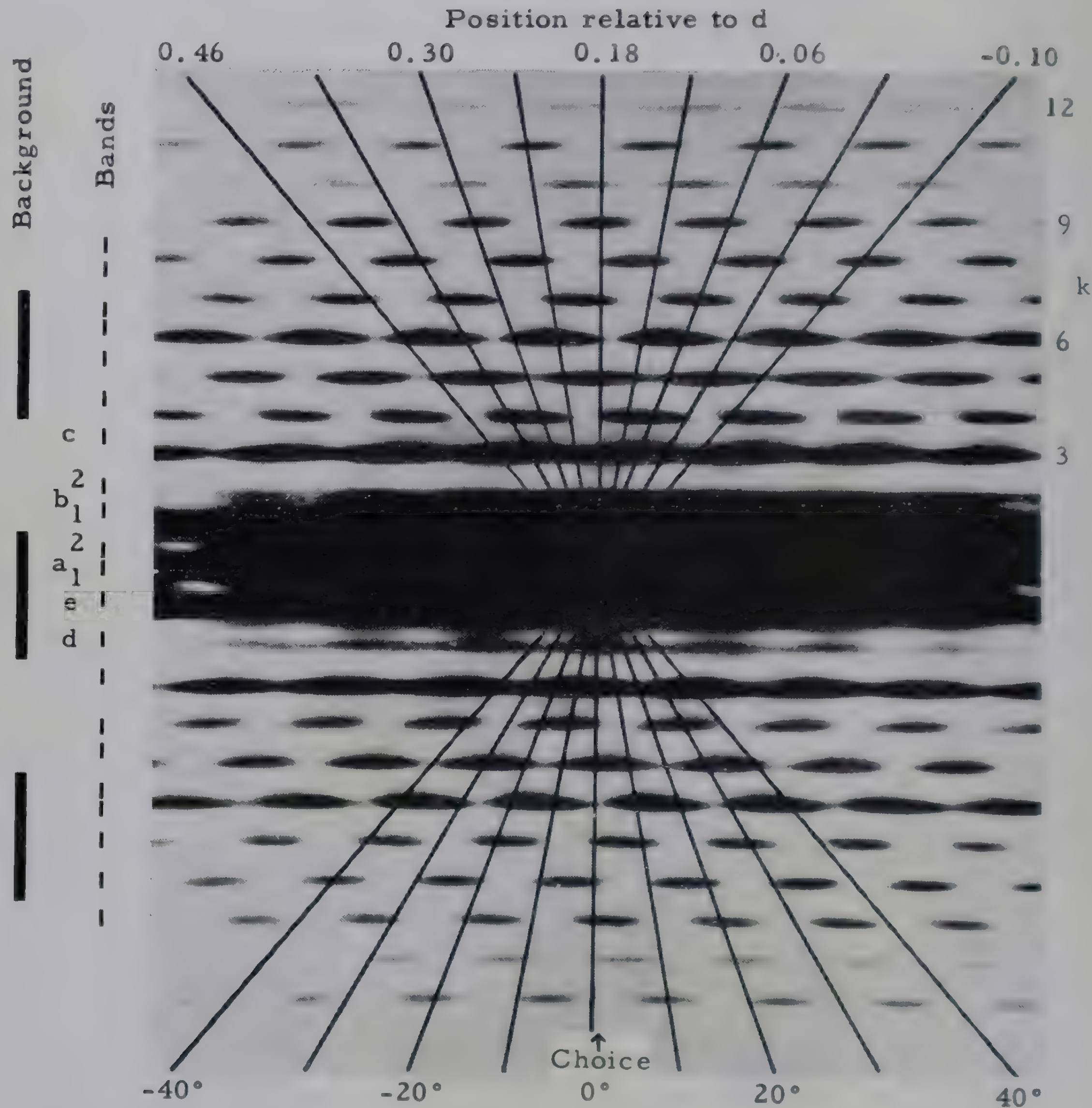
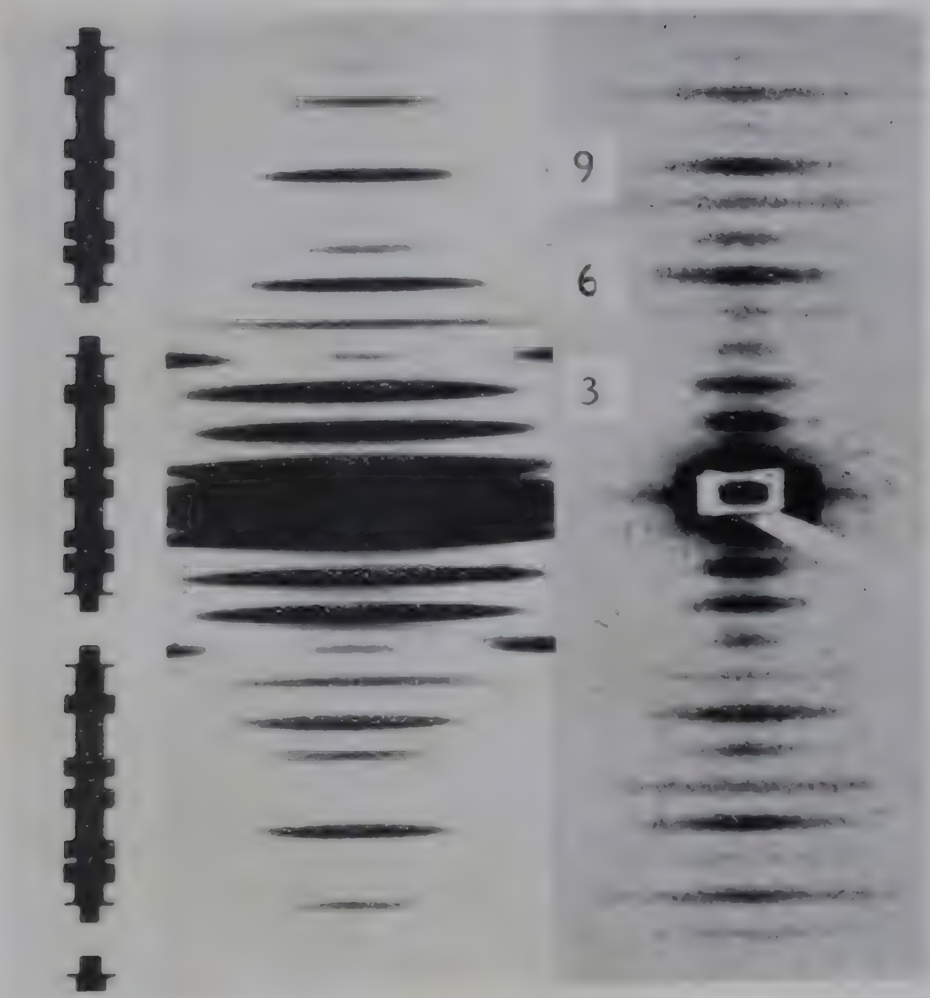


FIG. 2

Illustrating the method of canvassing background positions, relative to bands, for the moist fibril. In the models on the left, horizontal widths of blocks determine relative weighting of background and bands. Vertical lengths of background blocks determine the fraction of a macroperiod covered. Angles of projection on the lower border of the optical diffraction pattern measure the background centre's position, given at the top relative to the *d* band's centre as origin. Increase of the lateral separation between background and bands permits greater range of positional testing, as had been done prior to this final trial, whose best solution is indicated by 'choice'.

Model *Diffraction* *Patterns*

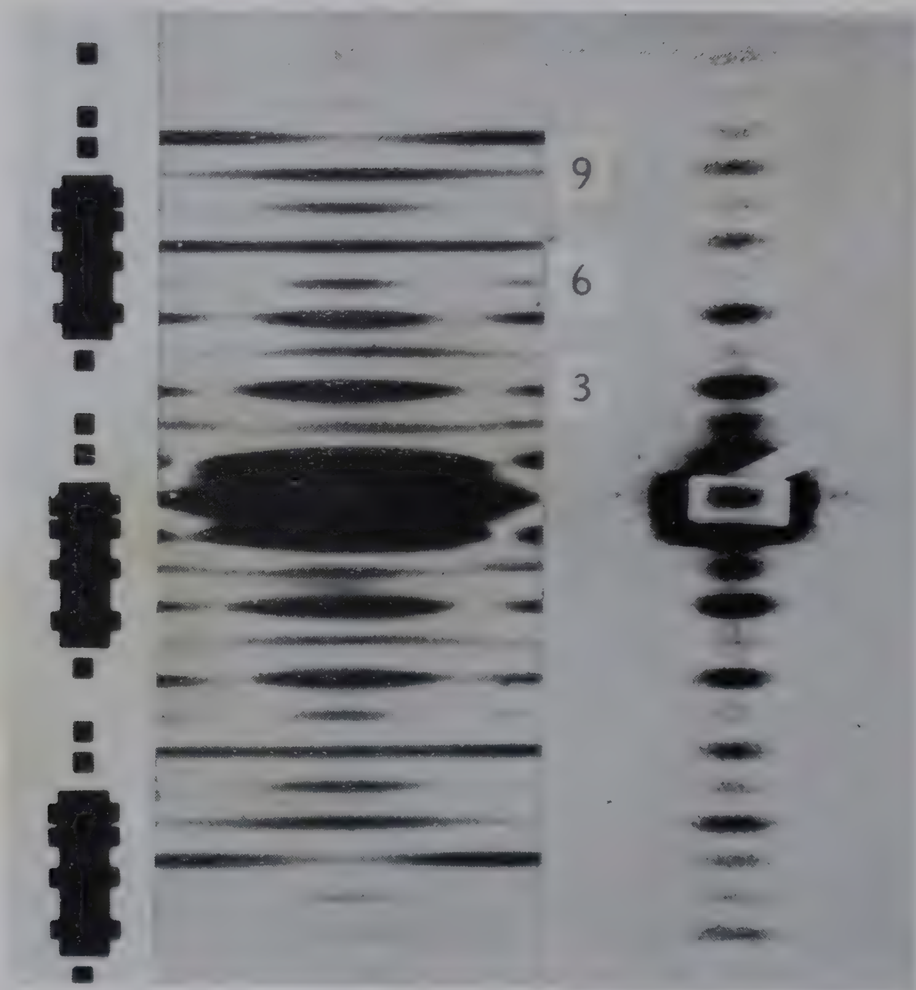


Optical *X-Ray*

FIG. 3

Comparison of the model for the dry fibril with corresponding optical and X-ray diffraction. Bands are the extra widths applied to backgrounds, with bands *d* and *e* at background edges. In this model background covers 0.78 of the macroperiod, with centre at 0.62 relative to *d* and horizontal width 1.5 times that added by bands.

Model *Diffraction* *Patterns*



Optical *X-Ray*

Fig. 4

Comparison of the model for the moist fibril with corresponding optical and X-ray diffraction. Here, background covers 0.54 of the macroperiod, with centre at 0.18 relative to *d* and horizontal width 2.5 times that added by bands.

multiple of fifths of the macroperiod. Inspection of electron optical data suggested approximately four-fifths for background coverage and further refinements were canvassed as follows.

The problems of selecting the final background length, its position relative to bands, and a reasonable relative weighting of bands and background involved tedious procedures, which were facilitated by the optical method exemplified by Fig. 2. This figure is, however, for the more crucial similar problem encountered with wet fibrillar models. Background and band portions of the model were separated laterally and given different widths corresponding to weighting. Background vertical length was varied from trial to trial. The resulting optical diffraction was then examined at several angles, as shown, to inspect for the best manner of projection of background upon the bands.

The solution finally selected for the model reproducing dry collagen diffraction is shown in Fig. 3. Note that optical and X-ray patterns should, in principle, be compared only directly at the individual line centres; the reasons for variations in horizontal line lengths are different in the two cases. The agreement is good, although doubtless it could be improved by incorporating further detail into the models, but this seems unwarranted at present.

In considering the corresponding problems for moist collagen, one has practically no secure help from electron microscopy. We have considered this case, nevertheless, making the primary simple but reasonable assumption that the principal bands are still present as slightly denser levels than their surroundings, and located at essentially the same relative positions as in the dry situation. The pronounced alteration of small-angle X-ray order intensities (with odd orders strong, even ones weak relative to neighbours, which continues out to the eleventh order, *cf.* Fig. 4), suggests a background which is now only about half the macroperiod in length.

One of the principal problems encountered in locating the background was that of having the strong sixth line contribution of the bands strongly opposed by the background diffraction, while yet maintaining moderately strong orders of indices 5, 7, 9 but not 11. It was also necessary to put a moderate amount of intensity into the second order, which neither the bands nor a background of exactly half-period length could accomplish. When these conditions were met, a reasonable satisfaction could be taken in the final result, shown in Fig. 4.

MAGNITUDES OF ELECTRON DENSITY VARIATIONS

In Fig. 5 the models thus far derived for the moist and dry fibrils are compared. Dotted lines indicate the result of using phases calculated from the models, along with X-ray amplitudes, to form Fourier plots of electron density projected on the fibrillar axis. It will be noted that the curve for dry collagen is very similar to densitometer traces of Nemetschek, Grassmann and Hofmann (1955, their Fig. 1) for kangaroo tendon, stained, however, with phosphotungstate.

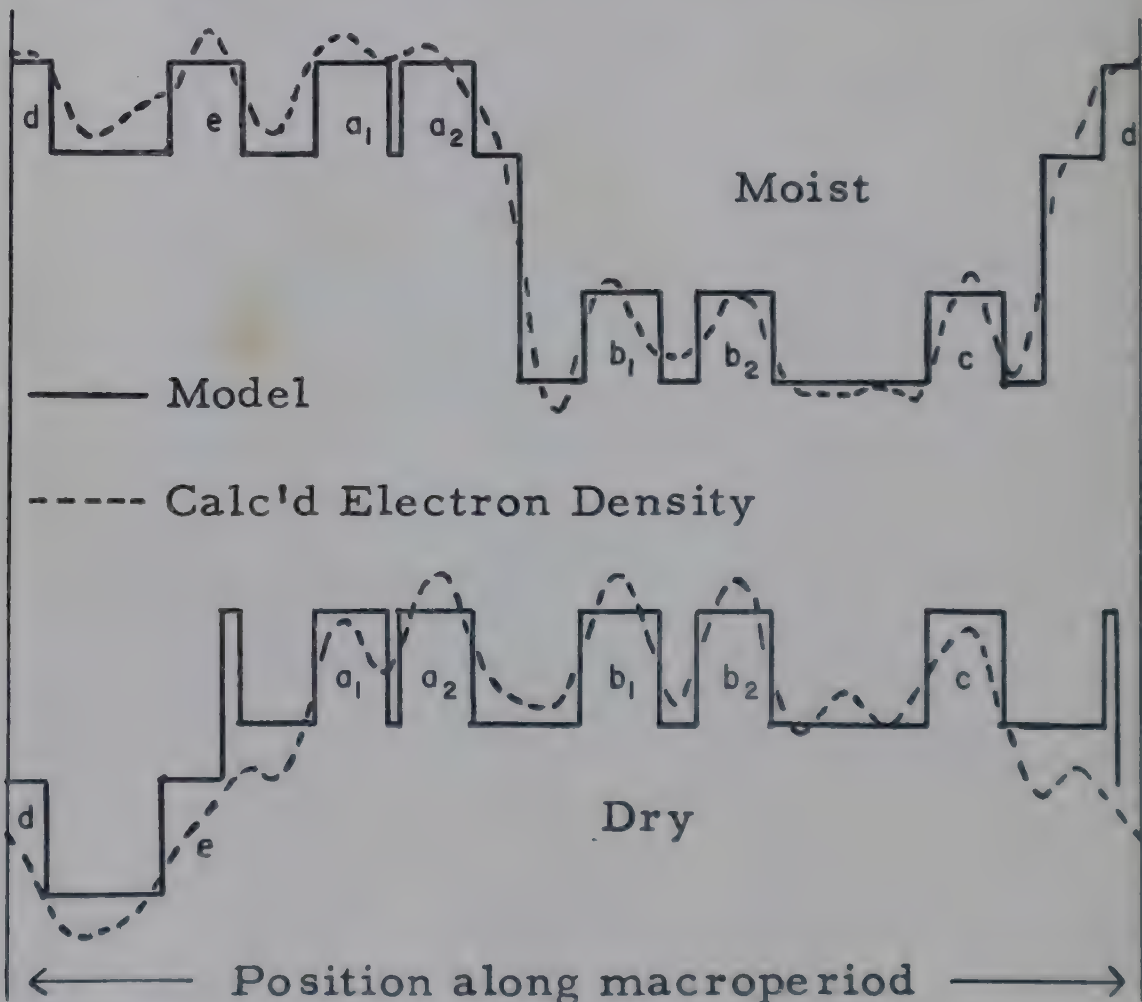


FIG. 5

Comparison of the models for dry and moist fibrils. Dotted curves show electron density projections obtained from calculated phases and X-ray amplitudes. Ordinates express axial projections of electron density in arbitrary units. One macroperiod is represented horizontally.

In the following discussion it is important to realize that the ordinates of Fig. 5 are in arbitrary units, and that one cannot at present estimate the absolute scale of either plot or tell the relative scales of

the two plots. One may arrive at qualitative suggestions regarding this matter from consideration of what is known of the molecules involved in fibril formation.

Four separate lines of attack (Rich and Crick, 1955; Cowan, McGavin and North, 1955; Ramachandran, 1956; and Bear, 1956) have provided convincing evidence of the 3-strand, coiled-coil nature of the main polypeptide chain configuration of the collagen molecule. There are several similar structures now under consideration, aimed at disclosing which may singly or in combination provide the valid molecular organization. Since there is no wide-angle diffraction evidence of other significantly different kinds of polypeptide configuration in native collagen, one expects the favoured kinds of 3-strand structure to extend throughout the molecular length, except when distortions are introduced as described below.

Electron-optical (Gross, Highberger and Schmitt, 1954) and physico-chemical (Boedtker and Doty, 1955) evidence also indicates that the collagen molecule is a thin particle (diameter 14 Å.) of great length (2500 to 3000 Å.). The molecular length is about four times the 600 to 700 Å. fibrillar macroperiod experienced with dry to moist native specimens as measured by X-rays. It follows that some sort of extensive relative axial displacement of molecules, from exact transverse register of their ends, is necessary to explain development of a native fibrillar macroperiod shorter than individual molecular lengths. Evidence of the way this occurs may develop when small-angle, near-equatorial diffraction is more thoroughly explored (North, Cowan and Randall, 1954).

Tomlin (1956) has described the most specific proposals thus far for the nature of and reasons for this axial displacement. Of chief importance to the problem of axial distribution of matter are his suggestions regarding 'interstitial' overlapping of molecular ends, or the alternative 'defects' arising where large gaps may be left between ends. Tomlin's bands and interbands arise from these end effects and individually extend over appreciable contiguous fractions of the macroperiod. They are not comparable to the narrower separable and distributed stratifications usually meant by these terms, being more like our backgrounds. One of us (Bear, 1952) has suggested that the finer bands are caused by compositional differences, with residues of larger molal volumes preferentially congregated at bands.

Electron-optical replica studies show that moist fibrils are smooth (Gross and Schmitt, 1948), and essentially similar conclusions follow from studies of small-angle meridional line lengths (Bolduan and Bear, 1951). Molecular distortions leading to fibril corrugation are largely 'ironed' out by moisture, leading to a more perfect structure (Rougvie and Bear, 1953). If equal numbers of molecules pass through equal cross-sections at all fibrillar levels, or even if overlaps or defects cause shifting of water around within the fibril, relatively small differences can be expected in electron density projected on the axis of a smooth fibril. We estimate about 3 to 4 per cent difference between interbands and bands, with the latter being the denser, assuming that the two differ only compositionally in an extreme way. Molecular end effects could add to this difference a small amount.

Dry fibrils are, as is well known, corrugated by diametral variations. Intrinsic material densities, assuming even extreme compositional differences between bands and interbands, can be shown to be very little different from the average over-all experimental density of 1.41 found by Pomeroy and Mitton (1951) for dry calf hide. The axially projected differences in density, which are important in causing meridional small-angle X-ray diffraction, are, however, large because of the diametral variations remaining after removal of water. Possible reasons for the diametral variations follow.

During drying, normal relaxed specimens show no appreciable change in the meridional wide-angle 2.86 Å spacing (Bear, 1944) which is now known to correspond to the axial projection per residue along single polypeptide chains, throughout the molecule in moist cases or along interband portions largely in dry instances. The most direct reason for assuming that the wide-angle diffraction of dry fibrils is produced largely by interbands, and is related little to bands, comes from the fact that electron stains and tanning agents have relatively little effect on wide-angle diffraction (Bolduan, Salo and Bear, 1951). Drying, staining and tanning do affect small-angle diffraction; for example, dehydration decreased the macroperiod from 660 Å. (moist) to 603 Å. (dry) in the kangaroo tail tendon studied by Rougvie and Bear (1953).

On the assumption that this axial contraction occurs chiefly at bands, one can show that as much as about 20 per cent variation in diameter between band and interband may be required if their

intrinsic densities remain nearly equal. This would correspond to electron density projection differences of around 40 per cent. Defects or overlaps because of molecular ends would occur at about one out of four molecules across an involved cross-section, to produce roughly 25 per cent variations in projected density or half that in diametral differences. Experimental fluctuations in diameter as high as 15 per cent have been reported (Nutting and Borasky, 1948).

In summary, there are two possible sources for the projected density variations disclosed in Fig. 5: compositional ones leading to intrinsic density or diametral differences, or effects arising from molecular ends. In any case, the smooth moist fibril's variations are probably of lesser magnitude than the sizeable ones encountered in the corrugated dry fibril. Absolute measures of densities and diameters would be useful in further interpretation of these factors.

THE SIGNIFICANCE OF THE 'BACKGROUNDS'

The backgrounds which were found necessary additions to the bands in forming satisfactory models are different in two respects for the moist and dry cases: (*a*) they are of different lengths, and, of still greater interest, (*b*) they apparently cover different sets of bands. Of the two differences, we believe the former to be best established; the latter is more sensitive to assumptions involved in derivation of the moist fibril models.

The length of 0.54 for background of moist fibrils agrees with indications from Patterson plots, as described above. In the dry material, however, Tomlin and Worthington (1956) employed a much shorter length of 0.39 for a major block of their model, similar in significance to our background. This figure is close to two-fifths and would, therefore, result in little scatter to fifth and tenth orders. We believe that four-fifths (actually 0.78 was used here) yields a result more in line with electron-optical data. Note that Fig. 21 of Nemetschek, Grassmann and Hoffmann (1955), which summarizes their results in terms of band position measurements, shows a lack of bands between *d* and *e*, which region also corresponds to the minimum in our curve for density of dry material.

The actual background length used for the dry fibril may be somewhat arbitrary, dependent on the representation used for bands. Since the long background occurs over the region where bands are most concentrated, its addition to the model in effect corrects for inadequacies of detail in the assumed bands.

Tomlin and Worthington added further details to their dry model which, in spite of incorrect assumption of a plane of symmetry, yielded results whose agreement with experimental X-ray intensities for kangaroo tendon is expressed by a residual of 0.22. Our model, with correct symmetry, gave calculated intensities which, when scaled to and compared with their reported X-ray intensities in the same way, gave a better residual of 0.20. Similar comparisons between moist fibril models yielded residuals of 0.21 for theirs, 0.18 for ours, an expression essentially of improvement secured by adding electron-optical information about bands. Our procedures did not include extensive attempts to minimize residuals as such but did include optical canvassing of structures as described above.

We believe that it is important to consider the finer band details, so that backgrounds may be selected with greater accuracy relative to both length and location. When this is done, the difference between both characteristics for backgrounds of moist and dry fibrils reveals their essentially different meanings, which otherwise would be overlooked.

The background of the dry fibril may be said to disappear when water is added, causing instead appearance of a new background which may be at a very different place. Cohen (1954) found that tension alone causes dry kangaroo tendon (at a macroperiod of 700 Å) to yield a pattern resembling that of moist samples, showing the alternation of line intensities which requires the new background. Since both hydration and tension may be expected to straighten collagen molecules, thus removing distortions at bands, the interpretation of the above facts would be that the background of dry fibrils is merely a diffused base to the bands resulting from distortions produced by dehydration. It is then apparent that this distortional background may be similar to the modulating function which Burge and Randall (1955) found it necessary to introduce in relating band structure to X-ray small-angle diffraction.

The meaning of the background in the moist fibril is more difficult to determine. There are perhaps three chief possibilities: (1) certain bands, d, e, a_1 and a_2, may, even when moist, retain more persistently than others a degree of distortion which slightly enlarges the fibrillar diameter in their neighbourhood to result in greater material content and electron density projection on the axis; (2) these same bands may contain intrinsically more electron-dense residues than do b_1, b_2 and c; and (3) the background may be

related to the locations where molecular ends overlap ('interstitial' segments of Tomlin), or the $b_1 - b_2 - c$ trough may represent places where molecular ends are pulled away from contact ('defects' of Tomlin).

One cannot clearly decide between these causes at the moment, and indeed more than one may be operative. Relative to (2) above we have some preliminary evidence that partitioning of arginine and lysine between $d - a_2$, and $b_1 - c$ locations, respectively, could be at least a partial cause of their density differences. This work was done with optical methods which simulated an experiment of Bolduan, Salo and Bear (1951). These authors found that that calgon staining of untreated or deaminated kangaroo tendon gave preparations in which, respectively, chiefly arginine plus lysine or arginine alone were tagged, with accompanying changes in small-angle line intensities.

CONCLUSION

In approaching the problem of electron density distribution from small-angle X-ray diffraction and electron-optical information, one naturally expects that the elimination of water will facilitate examination of the protein component alone. The results of the two methods applied to dry samples can be said thus far to have provided maps of material distribution expressed as band and interband locations or plots of electron density along the typical fibril. These remain, however, rather non-specific with respect to interpretations in terms of residue or molecule locations, which can only be very roughly surmised by indirect arguments of the type given in this paper.

One of the chief disadvantages of normal, relaxed dry material turns out to be the diffusion of structure overlaying the basic band and interband distribution, expressed by the distortional background covering much of each macroperiod. When this is removed by hydration (or tension) one then has remaining smaller density variations, but ones, nevertheless, which may be more significant for determination of how collagen molecules, with their residue sequences, are distributed along the fibril.

Particular interest attaches to the meaning of the shorter background herein indicated in moist (or extended dry) fibrils to be located differently from the distortional one of relaxed dry fibrils.

This short background is largely responsible for the marked alternation of small-angle line intensities characteristic of moist specimens of collagen from vertebrate animals. It has been observed even with *Thyone* collagen (an echinoderm, 'sea cucumber', studied by Marks, Bear and Blake, 1949), whose pattern after dehydration is, nevertheless, significantly different from the commoner collagens with respect to intensity distribution. Thus, the intensity alternation, related to the shorter background, probably reflects a rather fundamental feature of the fibrillar structure of a wide variety of collagens. The importance of this phenomenon for general fibrillar structure of collagens makes it worthy of further detailed study.

In this paper we have given theoretical and experimental evidence supporting the following views regarding the distribution of matter along the collagen fibril:

(1) Axial electron density projections, bridging electron-optical and meridional small-angle X-ray diffraction data, are presented for moist and dry kangaroo tendon fibrils. In addition to properly located major band densities, one must add 'backgrounds' which cover different fractions of the macroperiod and may have different axial locations in the moist and dry cases.

(2) In the dry fibril background represents a distortional diffusion of structure at the concentrated band region underlying bands from *e* through *a*, *b* and *c* to *d*. Moisture removes this background as the molecules become straightened.

(3) With distortional background removed, a shorter background apparently covering bands *d*, *e*, a_1 and a_2 is disclosed. This background is of uncertain significance, although reasons are cited leading to the belief that it represents a fundamental feature of fibril structure, found widely in collagens.

GROUP DISCUSSION

Dr. Grassmann was pleased to hear that the chemical evidence and Dr. Bear's results were essentially in agreement. He asked if the background Dr. Bear had described might be due to amorphous matter. He also commented on the fact that even in the dry state the position of the bands is constant but the intensities are not.

Dr. Bear restated the discussion in the text of his paper relative to the

possible differences in the meanings of the backgrounds in moist and dry fibrils. In the sense that amorphous matter is distorted matter, a general distortional spreading of material about bands is one possible explanation of the backgrounds, invoked as most likely for the dry fibril. Chemical explanations seem more probable for the moist fibril. Relatively minor features of band structure have not been considered, but in a sense the assumption of background partially compensates for this.

Dr. Fitton Jackson said that Burge (1956) at King's College had calculated from a large number of observations of the low-angle X-ray diagram of dry fibres that there was not more than a 14 per cent difference in diameter between band and interband.

Dr. Neuberger asked if glutamine and asparagine have electron densities similar to lysine and arginine, and if this might account for discrepancies between the calculated pattern and the diagram observed.

Dr. Bear said that glutamic and aspartic acids, and their amides, have relatively similar electron densities, but lysine and arginine differ appreciably. Recognition of this fact led to consideration of the possibility that a specific distribution of basic side chains might be involved in the 'background effect' of moist fibrils. A specific distribution of the acid, amide or other residues might also exist, but its recognition by X-ray methods would be more difficult.

CHAIRMAN'S SUMMING-UP

R. E. TUNBRIDGE

In concluding this symposium it would be not only impossible but presumptive for a chairman even to attempt to summarize our deliberations. The communications, and the approved summaries of the discussions, are to be published, but, however excellent the final report, and I am sure that it will make good reading, it will miss something of the atmosphere of the symposium which only those who have been present have experienced.

Definitions are always difficult, even when one is dealing with a single language. It is not surprising therefore, that, in a rapidly developing field of knowledge and one embracing the most diverse techniques, difficulties have arisen concerning the nomenclature of the different preparations of collagen. The term acid soluble collagen for example has been variously interpreted as citrate soluble collagen, acid soluble collagen, or acid soluble collagen in buffered solution. It is unfortunate that in our attempts to simplify the nomenclature of the different preparations of collagen we were unable to reach complete agreement and that no agreed recommendation can be put forward by the group. Nevertheless, a considerable measure of understanding and of agreement has been reached which must inevitably be of value. Agreement was more easily reached concerning elastic tissue. This may have been due in part to the more limited interest as yet of this aspect of the connective tissue problem.

Professor Astbury, in his opening remarks, drew attention to a number of points which have been to the fore in all our discussion: the increasing importance of chemical analysis and the fact that the chemistry of collagen has temporarily at any rate, passed from the physical chemist to the analytical chemist; the difficulty in obtaining pure extracts and the need to account for the small percentage of carbohydrate; the value of studying the growth of tissues as well as the mature fibres and, finally, stress was laid on the existence of a family of collagens and the possibility that elastin may yet prove to be an intimate member of the family.

Comparative studies of animal tissues have indicated the variety

of collagen fibrils and possibly of collagens. The variation in the proportions of neutral and insoluble collagen in different tissues with age and at the different stages of response to carrageenin, are merely further proof of the existence of a variety of collagens. The chemical analyses of Bowes and her colleagues, and of Grassmann for collagen, and of Partridge for elastin, and the work of Grassmann on the amino-acid sequences, are brilliant achievements but they have not answered the problem as to what significance should be attached to the finding of polysaccharide and the anomalous protein fraction. There is still no agreement whether these are impurities, due to technical imperfections, and Neuberger stressed the importance of allowing for the presence of plasma proteins in the analysis of dermis. Much evidence has been brought forward indicating the possible role of mucopolysaccharides in fibril formation both intra- and extra-cellularly. Fitton Jackson's excellent illustrations have demonstrated the possibility of intracellular fibril formation, and Schwarz and Grassmann have revealed the possible intricacies of even the collagen fibrils as a result of silver staining techniques applied to treated material studied with the electron microscope.

Reference has constantly been made to the need for caution in employing similar terms for phenomena occurring at different levels of magnitude, molecular, macro-molecular, fibril and fibre. Further, the danger of relying upon a single method of estimation and the need to check and recheck different methods were repeatedly emphasized.

The complexities of the structure, composition and function of collagenous and allied fibrils remains all too apparent, but a much greater understanding of one another's difficulties and objectives has resulted from this symposium. 'In understanding is knowledge and through knowledge cometh truth.'

Finally I should like to express on behalf of C.I.O.M.S. appreciation of your ready acceptance of the invitation to be present and to take part in this symposium. I should also like to express on your behalf our thanks to Dr. Delafresnaye and to Mrs. Tausig for their constant attention to our wants, for their unfailing kindness and consideration, and for the very efficient organization of this Symposium.

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